IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of:
Figdor et al.

Application No.: 10/625,202 | Confirmation No.: 1242

Filed: July 23, 2003 | Art Unit: 1648

For: COMPOSITION AND METHOD FOR MODULATING DENDRITIC CELL-T CELL INTERACTION | Examiner: M. G. Hill

APPEAL BRIEF UNDER 37 C.F.R. § 41.37

MS Appeal Brief - Patents
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

This Appeal Brief is submitted in response to the Final Office Action mailed October 6, 2009, and in further response to the Advisory Action dated January 5, 2010, in support of the Pre-Appeal Brief and Notice of Appeal filed on February 4, 2010, and in response to the Notice of Panel Decision from Pre-Appeal Brief review mailed March 12, 2010. A request for a one-month extension of time and appropriate fee are submitted concurrently herewith.

This Brief has the following appendices:

Claims Appendix

Appendix A: Copy of claims 1, 3, 4, 6, 7, 19 and 23-27 involved in this appeal;

Evidence Appendices

Appendix B: Copy of the Final Office Action mailed October 6, 2009;

Appendix C:	Copy of the Advisory Action mailed January 5, 2010;
Appendix D:	Copy of Ingulli, et al., J. Exp. Med., vol 185, 2133-2141 (1997);
Appendix E:	Copy of Steinman, *Cell*, vol. 100, 491-494 (2000);
Appendix F:	Copy of Janeway et al. (2001), Immunobiology, 5th edition, pages 20-21; and
Appendix G:	Copy of Pereira et al., J. Immunother. 30:705-714 (2007).

Related Proceedings Appendix

None.

(i). REAL PARTY OF INTEREST

Appellants respectfully advise the Board that the real party in interest in the above-identified patent application is Katholieke Universiteit Nijmegen, a university organized and existing under the laws of the Netherlands, and having an office and place of business at Philips Van Leydenlaan 25, NL-6525 EX Nijmegen, Netherlands, which is the assignee of this application. Additionally, Alexion Pharmaceuticals, Inc., a corporation organized and existing under the laws of Delaware and having an office and place of business at 352 Knotter Drive, Cheshire, CT 06410, is the exclusive licensee of this application.

(ii). RELATED APPEALS AND INTERFERENCES

Appellants respectfully advise the Board that there are no other appeals or interferences known to appellants, their legal representative, or their assignee that will directly affect or be directly affected by or have a bearing on the Board's decision in the pending appeal.

(iii). STATUS OF CLAIMS

Claims 1, 3, 4, 6, 7, 19 and 23-27 are rejected in this application. Claims 2, 5, 8-18 and 20-22 were previously canceled. Claims 1, 3, 4, 6, 7, 19 and 23-27 are pending and on appeal. Claims 1, 3, 4, 6, 7, 19 and 23-27 were finally rejected in the Final Office Action dated October 6, 2009. Claim 1 is an independent claim; all other pending claims depend from claim 1 or another claim depending from it. No claims have been allowed. It is to be noted that the Notice

of Panel Decision from Pre-Appeal Brief Review and the Advisory Action both fail to indicate that claim 6 is pending. It is urged that such is in error. The Final Office Action of October 6, 2009 failed to indicate the status of claim 6 on the summary page but did properly refer to claim 6 in the rejection on page 2. It is noted that Applicant never canceled claim 6 and claim 6 was never withdrawn by the Examiner. It is urged that claim 6 is pending and is on appeal.

(iv). STATUS OF AMENDMENTS

Appellants have not submitted any amendment pursuant to 37 C.F.R. § 1.116 or in the reply to the October 6, 2009 Final Office Action (hereinafter "Office Action") or the January 5, 2010 Advisory Action (hereinafter "Advisory Action"), from which this appeal is being sought.

(v). SUMMARY OF CLAIMED SUBJECT MATTER

Commensurate with independent claim 1, an exemplary embodiment of the present invention is directed to a method for reducing a T-cell mediated immune response in a non-HIV infected animal by inhibiting an interaction between a dendritic cell and a T cell. The method includes administering to an animal in need of reducing the T-cell mediated immune response an antibody which binds to a protein with the amino acid sequence of SEQ ID NO: 2 (DC-SIGN) on the surface of a dendritic cell. Upon administration, the anti-DC-SIGN antibody reduces one or more interactions between a dendritic cell and a T cell thereby reducing the T-cell mediated immune response in the animal. *See, e.g.*, page 5, line 27 – page 7, line 21.

As required by 37 C.F.R. § 41.37(c)(1)(v), a concise explanation of the subject matter defined in the independent claims involved in the appeal is provided herein. Appellant notes that representative subject matter is identified for these claims; however, the abundance of supporting subject matter in the application prohibits identifying all textual and diagrammatic references to each claimed recitation. Appellant thus submits that other application subject matter, which supports the claims but is not specifically identified above, may be found elsewhere in the application. Appellant further notes that this summary does not provide an exhaustive or exclusive view of the present subject matter, and Appellant refers to the appended claims and their legal equivalents for a complete statement of the invention.

(vi). GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL

The following grounds of rejection are to be reviewed on this appeal:

Whether the rejection of claims 1, 3, 4, 6, 7, 19 and 23-27 under 35 U.S.C. § 112(1) is proper.

(vii). ARGUMENT

The Rejection under 35 U.S.C. §112, first paragraph

Claims 1, 3, 4, 6, 7, 19 and 23-27 are finally rejected under 35 U.S.C. § 112, first paragraph as failing to comply with the enablement requirement. Appellants respectfully traverse this rejection and request that it be overturned for at least the reasons set forth below.

A. The Examiner has not met his burden of establishing a prima facie case of lack of enablement.

The Examiner asserts that Applicants' argument based on MPEP § 2164.02 that *in vitro* examples are enough to provide enablement when there is good correlation is not persuasive because the section refers to small pharmaceutical molecules not antibodies, and the method requires binding to a cell and then triggering further action in the form of a reduced immune response. The Examiner further asserts that the examples in the instant specification and the prior art do not show this function. *See,* Office Action, the paragraph bridging pages 3 and 4.

Applicants assert that *in vivo* efficacy data are not required to enable an *in vivo* use and that *in vitro* test results are generally predictive of *in vivo* testing results. *See,* MPEP § 2164.02; and *Cross v. Iizuka*, 753 F.2d 1040, 1050 (Fed. Cir. 1985). Scientifically sound explanations, backed by *in vitro* testing, are widely accepted as sufficient evidence to support claims drawn to subject matter commensurate in scope with that support. *See, In re Brana*, 51 F.3d 1560 (Fed. Cir. 1995). The instant application provides several examples, including *in vitro* data derived from cell based assays, to support the claimed subject matter. For example, Example 2 demonstrates that an antibody against DC-SIGN can inhibit the interaction between DC-SIGN (on the surface of dendritic cells) and an ICAM receptor (on the surface of T cells). *See, e.g.*,

page 20, line 23 – page 21, line 2 and Figures 2A and 2C. Example 6 further demonstrates that: (1) anti-DC-SIGN antibodies prevented the clustering of dendritic cells with ICAM-3-expressing K562 cells (Figure 6B); (2) anti-DC-SIGN antibodies inhibited the clustering of dendritic cells with PBLs (which include T-cells) (Figure 6C); and (3) most importantly, anti-DC-SIGN antibodies inhibited the activation of T-cells when T-cells were mixed with dendritic cells (Figure 6D), as discussed in Example 6 and Example 7. These assays are representative of what occurs *in vivo*, i.e., there are interactions between dendritic cells and T cells which initiate an immune response. *See, e.g.*, page 19, lines 1-10 of the instant application. This interaction is mediated by DC-SIGN expressed on the surface of dendritic cells and an ICAM receptor expressed on the surface of T-cells. *See, e.g.*, page 1, lines 9-12 of the instant application. The application also teaches and enables reducing an immune response by inhibiting an interaction between dendritic cells and T cells by blocking the interaction between DC-SIGN (expressed on dendritic cells) and an ICAM receptor (expressed on T-cells). Therefore, the application teaches and enables the method as recited in independent claim 1. A person of ordinary skill in the art can make and use the claimed methods based on the *in vitro* data and other teachings of the specification, without undue experimentation.

Applicants have submitted additional evidence demonstrating that one of skill in the art would expect that the *in vitro* evidence provided in the specification is representative of what occurs *in vivo*. For example, Ingulli, et al., *J. Exp. Med.*, vol 185, 2133-2141 (1997) ("Ingulli," previously submitted as Exhibit A attached to the response dated April 28, 2008, attached herein as Appendix D) demonstrates that antigen-bearing dendritic cells directly interact with naive antigen-specific T cells (Ingulli, abstract). This result is consistent with *in vitro* experiments suggesting that dendritic cells are initiating antigen presenting cells (APCs) for T cell responses (Ingulli, page 2133, left column). Applicants cite Ingulli to demonstrate that in this area, *in vitro* results are consistent with *in vivo* results. Similarly, Steinman, *Cell*, vol. 100, 491-494 (2000) ("Steinman," previously submitted as Exhibit B attached to the response dated April 28, 2008, attached herein as Appendix E) was submitted to show that in this area, several *in vivo* studies have corroborated previous *in vitro* results (Steinman, page 492, right column).

Although the specification has not provided *in vivo* data showing an actual reduction of immune response *in vivo*, it is textbook knowledge that T cell activation requires the interaction between dendritic cells and T cells. *See, e.g.*, Janeway et al. (2001), Immunobiology, 5th edition,

pages 20-21 ("Janeway," previously submitted as Exhibit 1 attached to the response dated June 8, 2009, attached herein as Appendix F). Janeway teaches that T-cells can be activated by a combination of two signals – an antigen and a dendritic cell. The pending claims are directed to inhibiting the T-cell-dendritic cell interaction, thereby preventing the activation of the T-cell, e.g., a T-cell mediated immune response. In view of the *in vitro* data provided by the present application, and the additional evidence provided by Ingulli, Steinman and Janeway, one of ordinary skill in the art would have had no reason to question whether the *in vitro* data provided by the application indeed correlate with *in vivo* efficacy.

Furthermore, additional publications in the field indicate that when an anti-DC-SIGN antibody was administered *in vivo*, the antibody successfully bound to DC-SIGN expressed on the surface of dendritic cells *in vivo*. For example, Pereira et al., J. Immunother. 30:705-714 (2007) ("Pereira," previously submitted as Exhibit 2 attached to the response dated June 8, 2009, attached herein as Appendix G) reports the specific targeting of DCs *in vivo* in a nonhuman primate model using antibodies directed against DC-SIGN (see, summary, page 705). As such, Pereira confirms that the *in vitro* data of the present application are reliable and the application correctly predicted the *in vivo* effect. One of ordinary skill in the art would have had no reason to question whether the *in vitro* data provided by the application indeed correlate with *in vivo* efficacy.

Further, Applicants submit that the Examiner has the burden to provide reasons for a conclusion of a lack of correlation with *in vitro* testing or an *in vivo* animal model. *See,* MPEP 2164.02. In this case, the Examiner has merely asserted that the level of unpredictability in the art is high and on this basis concludes that one skilled in the art would not associate *in vitro* efficacy with *in vivo* treatment. The Examiner has not provided any specific reasons to doubt that the *in vitro* data correlate with *in vivo* efficacy. The Examiner has not provided any evidence to suggest that the instantly claimed methods would not be operative with respect to reducing T-cell mediated immune response *in vivo*, thereby failing to meet the burden of establishing a *prima facie* case of lack of enablement.

Applicants point out that the scope of enablement only needs to bear a "reasonable correlation" to the scope of the claims. *See, e.g., In re Fisher*, 427 F.2d 833, 839, 166 USPQ 18, 24 (CCPA 1970); MPEP 2164.08. There is no requirement in U.S. patent law that the claimed

invention must work 100% of the time under all conceivable circumstances. "All that is necessary is that one skilled in the art be able to practice the claimed invention, given the level of knowledge and skill in the art." MPEP 2164.08. Applicants submit that the data presented in the specification more than satisfy the "reasonable correlation" of the *in vitro* working examples with the claimed method as required by case law.

Regarding the Examiner's arguments, the quoted section of the MPEP (MPEP § 2164.02) itself is not directed to small molecules. Even if the particular case law being cited (*Cross v. Iizuka*, 753 F.2d 1040 (Fed. Cir. 1985)) involves a small molecule, the MPEP makes no distinction between a small molecule and a large molecule. In addition, Applicants submit that the distinction drawn by the Examiner between a small molecule and a large molecule (e.g., an antibody) is irrelevant to the enablement rejection. It is well known that antibodies bind to their antigens with exquisite specificity. The specification demonstrates that the disclosed antibodies bind to DC-SIGN and inhibit the interaction between DC-SIGN on the surface of dendritic cells and an ICAM receptor on the surface of T cells. The Examiner did not explain why the type of molecule, e.g., an antibody as compared to a small molecule, would make any difference in evaluating enablement.

In addition, Applicants point out that the Examiner has incorrectly characterized the claimed method by stating that "the method requires binding to a cell and then *triggering further action* in the form of a reduced immune response" (page 3, last line of the Office Action, emphasis added). In the claimed method, an anti-DC-SIGN antibody binds to its target (DC-SIGN represented by SEQ ID NO: 2), thereby blocking the DC-SIGN from further interactions with a T-cell. With DC-SIGN blocked, the further actions (e.g., interaction with a T-cell) that are required for an immune response cannot occur, resulting in a decreased immune response. Thus, the binding of the antibody does not trigger further action as the Examiner asserts; to the contrary, it blocks further action. Accordingly, in the claimed method, an antibody may actually be better than a small molecule since the antibody may block an interaction between DC-SIGN and a T-cell more efficiently due to steric interference. All that is required is for the antibody to bind to DC-SIGN thereby blocking interaction of dendritic cells with T-cells.

B. The claimed invention directed to decreasing an immune response is differentiated by distinct method steps.

The Examiner asserts that "[t]he complexity of the art and of the currently claimed invention is that an antibody that binds SEQ ID# 2 can both increase and decrease the immune response. While applicant argues that the specification includes two different inventions, other than the description in the disclosure or the preamble in the claims, the difference in immune response in the methods [is not] differentiated by distinct method steps." *See,* Office Action, page 3, lines 12-16.

While the specification teaches that certain embodiments of the application relate to methods of increasing an immune response in an animal, the immune response is against a specific antigen by presenting an antigen (or one or more antigenic parts thereof) to dendritic cells in a form that can bind to the C-type lectin receptors on the surface of dendritic cells, which then process and present the antigen to the T-cells, thereby causing an immune response against the antigen (e.g., page 15, lines 3-26). The specification further teaches that "the antigen can be attached to (e.g. fused with or covalently bonded to) an antibody directed against the C-type lectins, preferably a monoclonal antibody such as AZN-D1 and AZN-D2 mentioned above; or to a part or fragment of such an antibody as described above" (e.g., the paragraph bridging pages 15 and 16). Briefly, an antibody may be attached to a specific antigen and this antibody-antigen conjugate may be used for targeting the antigen to dendritic cells, and subsequently inducing an immune response against the specific antigen (see, e.g., page 16, line 29-page 17, line 3).

By contrast, the claimed invention (e.g., claim 1) is directed to a method for reducing a T-cell mediated immune response in an animal by using a naked antibody that binds to DC-SIGN on the surface of a dendritic cell thereby blocking the interaction of the dendritic cell with a T-cell. Such method results in an inhibition of T-cell mediated immune activity. Contrary to the Examiner's assertion, one of skill in the art could readily differentiate these two inventions, which require different forms of an antibody (the first requires an antibody-antigen conjugate, whereas the second uses a naked antibody) and cause different outcomes (the first causes an increase in a specific immune response, whereas the second causes a decrease in immune activity).

C. *The specification teaches the in vivo significance of the claimed method.*

The Examiner asserts that the specification does not teach the amount or type of reduced immune response, the significance *in vivo* or what that level of reduction produces. *See,* Advisory Action page 2.

Applicants assert that the specification does teach the amount and type of reduced immune response, the significance *in vivo* and what that level of reduction produces. *See,* for example, specification page 6, line 30 – page 7, line 21. Specifically, the specification teaches that anti-DC-SIGN antibodies bind to DC-SIGN, and inhibit the interaction between the DC-SIGN on the surface of dendritic cells and an ICAM receptor on the surface of T cells. The specification teaches that such interactions include the adhesion of T-cells to dendritic cells, for instance in dendritic cell-T-cell clustering and T-cell activation. These dendritic cell-T-cell interactions are involved in generating an immune response, such as primary sensitization/activation of T-lymphocytes, and co-stimulation of T cells; as well as processes such as chemical signaling, endocytosis and transepithelial transport. As disclosed in the specification, specific applications include, for example, preventing or inhibiting immune responses to specific antigens and immunosuppression, for instance to prevent transplant rejection and treatment of autoimmune diseases. Therefore, one of skill in the art would have understood the significance of the invention *in vivo* and what to expect from the *in vitro* examples.

Based on all of the above arguments, Appellants respectfully request that the Board overturn the §112 Rejection of claims 1, 3, 4, 6, 7, 19 and 23-27.

(viii). CONCLUSION

For the reasons given above, it is respectfully urged that the final rejection be reversed and that all pending claims be allowed.

Appellants authorize the Commissioner to withdraw the extension of time fee from Deposit Account 18-1945. If there are any other fees not accounted for, Appellants authorize the Commissioner to charge the fee to Deposit Account 18-1945.

If there are any questions after reviewing this paper, the Examiner is invited to contact the undersigned at (617) 951-7000.

Dated: May 5, 2010

Respectfully submitted,

/Ryan Murphey/

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(ix). CLAIMS APPENDIX

CLAIMS APPENDIX A

CLAIMS ON APPEAL

1. A method for reducing a T-cell mediated immune response in an animal by inhibiting an interaction between a dendritic cell and a T cell, comprising administering to an animal in need of reducing said immune response an antibody which binds to a protein with the amino acid sequence of SEQ ID NO: 2 (DC-SIGN) on the surface of a dendritic cell, wherein said antibody reduces one or more interactions between a dendritic cell and a T cell thereby reducing said immune response in the animal, and wherein the animal is not infected with HIV.

2. (Canceled).

3. The method of claim 1 wherein said animal is a mammal.

4. The method of claim 3 wherein said mammal is a human.

5. (Canceled).

6. The method of claim 1 wherein said antibody reduces adhesion between DC-SIGN and an ICAM receptor on the surface of a T cell.

7. The method of claim 6 wherein said ICAM receptor is selected from the group consisting of ICAM-2 receptors and ICAM-3 receptors.

Claims 8 - 18. (Canceled).

19. The method of claim 1 wherein said antibody is a monoclonal antibody.

Claims 20 - 22. (Canceled).

23. The method of claim 1 wherein said antibody is selected from the group consisting of i) an antibody produced by hybridoma ECACC accession number 99040818 and ii) an antibody produced by hybridoma ECACC accession number 99040819.

24. The method of claim 1, wherein said animal is in need of tolerance, immunotherapy or immunosuppression.

25. The method of claim 1, wherein said animal is suffering from an autoimmune disease.

26. The method of claim 1, wherein said animal is suffering from an allergy.

27. The method of claim 1, wherein the antibody is administered in combination with another compound selected from the group consisting of: immunosuppressants, immunomodulants, antibiotics, auto-antigens, allergens, anti-LF3A, Tumor Necrosis Factor (TNF), anti-viral agents, and CD4 inhibitors.

(x). EVIDENCE APPENDIX

EVIDENCE APPENDIX B
COPY OF THE FINAL OFFICE ACTION MAILED OCTOBER 6, 2009

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO	CONFIRMATION NO.
10/625,202	07/23/2003	Carl Gustav Figdor	ALXN-P02-089	1242

28120 7590 10/06/2009

ROPES & GRAY LLP
PATENT DOCKETING 39/41
ONE INTERNATIONAL PLACE
BOSTON, MA 02110-2624

EXAMINER	
HILL, MYRON G	

ART UNIT	PAPER NUMBER
1648	

MAIL DATE	DELIVERY MODE
10/06/2009	PAPER

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Office Action Summary	Application No.	Applicant(s)	
	10/625,202	FIGDOR ET AL.	
	Examiner	Art Unit	
	MYRON G. HILL	1648	

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE *3* MONTH(S) OR THIRTY (30) DAYS, WHICHEVER IS LONGER, FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☒ Responsive to communication(s) filed on *08 June 2009*.

2a)☒ This action is **FINAL**. 2b)☐ This action is non-final.

3)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

4)☒ Claim(s) *1,3,4,7,19 and 23-27* is/are pending in the application.

4a) Of the above claim(s) _____ is/are withdrawn from consideration.

5)☐ Claim(s) _____ is/are allowed.

6)☒ Claim(s) *1,3,4,7,19 and 23-27* is/are rejected.

7)☐ Claim(s) _____ is/are objected to.

8)☐ Claim(s) _____ are subject to restriction and/or election requirement.

Application Papers

9)☐ The specification is objected to by the Examiner.

10)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

11)☐ The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

a)☐ All b)☐ Some * c)☐ None of:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☐ Notice of References Cited (PTO-892)
2) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)
3) ☒ Information Disclosure Statement(s) (PTO/SB/08) Paper No(s)/Mail Date *6/8/09*.
4) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____.
5) ☐ Notice of Informal Patent Application
6) ☐ Other: _____.

DETAILED ACTION

This action is in response to the papers filed 6/8/09.

Claims 1,3,4,6,7, 19 and 23-27 are under consideration.

IDS

A signed and initialed copy of the IDS filed 6/8/09 is enclosed.

Claim Rejections - 35 USC § 112

The following is a quotation of the first paragraph of 35 U.S.C. 112:

> The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same and shall set forth the best mode contemplated by the inventor of carrying out his invention.

Claims 1, 3,4,6,7,9, 19 and 23-27 are rejected under 35 U.S.C. 112, first paragraph, as failing to comply with the enablement requirement. The claim(s) contains subject matter which was not described in the specification in such a way as to enable one skilled in the art to which it pertains, or with which it is most nearly connected, to make and/or use the invention.

Applicant argues that the disclosure teaches two distinct inventions: 1) reducing immune response by inhibiting interaction of DC-SIGN and T-cell and 2) a method to prevent HIV infection by inhibiting DC-SIGN and GP120 interaction. Invention 1 is not

drawn to treating HIV infection. Furthermore, citing Steinman (previously supplied) and the current application both teach that DC-SIGN can be stimulated to induce response against antigen. Also, the restriction in the parent case highlights the difference as claimed.

Applicant also argues that in vitro is predictive of in vivo responses and lists in vitro examples from the specification. And that Steinman and Janeway (Editor) teach that DC and T-cell interaction induce immune response. Applicant teaches Pereira et al. shows in vitro to in vivo correlation.

Applicant's arguments have been fully considered and not found persuasive.

The results in the specification or those shown in the art provided do not show the reduction of immune response in mammals by an antibody that binds to SEQ ID# 2.

The complexity of the art and of the currently claimed invention is that an antibody that binds SEQ ID# 2 can both increase and decrease the immune response. While applicant argues that the specification includes two different inventions, other than the description in the disclosure or the preamble in the claims, the difference in immune response in the methods is not is not differentiated by distinct method steps.

Applicants argument that in vitro examples are enough when there is good correlation is not persuasive. First, the portion of the MPEP quoted refers to small pharmaceutical molecules not antibodies, and that the method requires binding to a cell and then triggering further action in the form of a reduced immune response. As

previously stated, the examples and the prior art do not show this function. Applicants argument that Steinman and Janeway (Editor) induce immune response evidence the fact that the prior art does not enable the claimed invention. Also, Pereira et al. (not found attached as an exhibit) teach in vitro effect is predictive of in vivo.is not persuasive for the following reasons: it is not drawn to reducing an immune response, it uses antigen bound to antibody which is not required in the present claims, and it appears from the abstract that non-human testing was done to demonstrate their experimental situation.

As far as the restriction in the parent case is concerned, the examiner is not making a double patenting rejection and the claims in this application are examined on their merits.

The rejection is maintained.

Conclusion

No claim is allowed.

THIS ACTION IS MADE FINAL. Applicant is reminded of the extension of time policy as set forth in 37 CFR 1.136(a).

A shortened statutory period for reply to this final action is set to expire THREE MONTHS from the mailing date of this action. In the event a first reply is filed within

TWO MONTHS of the mailing date of this final action and the advisory action is not mailed until after the end of the THREE-MONTH shortened statutory period, then the shortened statutory period will expire on the date the advisory action is mailed, and any extension fee pursuant to 37 CFR 1.136(a) will be calculated from the mailing date of the advisory action. In no event, however, will the statutory period for reply expire later than SIX MONTHS from the mailing date of this final action.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to MYRON G. HILL whose telephone number is (571)272-0901. The examiner can normally be reached on M-Th and flex.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Larry Helms can be reached on 571-272-0832. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative or access to the automated information system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

/Mary E Mosher/
Primary Examiner, Art Unit 1648

/M. G. H./
Examiner, Art Unit 1648

EVIDENCE APPENDIX C
COPY OF THE ADVISORY ACTION MAILED JANUARY 5, 2010

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
10/625,202	07/23/2003	Carl Gustav Higdon	ALXN-P02-089	1242

28120 7590 01/05/2010

ROPES & GRAY LLP
PATENT DOCKETING 39/41
ONE INTERNATIONAL PLACE
BOSTON, MA 02110-2624

EXAMINER
HILL, MYRON G

ART UNIT	PAPER NUMBER
1648	

MAIL DATE	DELIVERY MODE
01/05/2010	PAPER

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Advisory Action Before the Filing of an Appeal Brief	Application No.	Applicant(s)	
	10/525,202	FIGDOR ET AL.	
	Examiner	Art Unit	
	MYRON G. HILL	1648	

--The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

THE REPLY FILED 04 December 2009 FAILS TO PLACE THIS APPLICATION IN CONDITION FOR ALLOWANCE.

1. ☒ The reply was filed after a final rejection, but prior to or on the same day as filing a Notice of Appeal. To avoid abandonment of this application, applicant must timely file one of the following replies: (1) an amendment, affidavit, or other evidence, which places the application in condition for allowance; (2) a Notice of Appeal (with appeal fee) in compliance with 37 CFR 41.31; or (3) a Request for Continued Examination (RCE) in compliance with 37 CFR 1.114. The reply must be filed within one of the following time periods:

   a) ☐ The period for reply expires _____months from the mailing date of the final rejection.

   b) ☒ The period for reply expires on: (1) the mailing date of this Advisory Action, or (2) the date set forth in the final rejection, whichever is later. In no event, however, will the statutory period for reply expire later than SIX MONTHS from the mailing date of the final rejection.

   Examiner Note: If box 1 is checked, check either box (a) or (b). ONLY CHECK BOX (b) WHEN THE FIRST REPLY WAS FILED WITHIN TWO MONTHS OF THE FINAL REJECTION. See MPEP 706.07(f).

Extensions of time may be obtained under 37 CFR 1.136(a). The date on which the petition under 37 CFR 1.136(a) and the appropriate extension fee have been filed is the date for purposes of determining the period of extension and the corresponding amount of the fee. The appropriate extension fee under 37 CFR 1.17(a) is calculated from: (1) the expiration date of the shortened statutory period for reply originally set in the final Office action; or (2) as set forth in (b) above, if checked. Any reply received by the Office later than three months after the mailing date of the final rejection, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

NOTICE OF APPEAL

2. ☐ The Notice of Appeal was filed on _____. A brief in compliance with 37 CFR 41.37 must be filed within two months of the date of filing the Notice of Appeal (37 CFR 41.37(a)), or any extension thereof (37 CFR 41.37(e)), to avoid dismissal of the appeal. Since a Notice of Appeal has been filed, any reply must be filed within the time period set forth in 37 CFR 41.37(a).

AMENDMENTS

3. ☐ The proposed amendment(s) filed after a final rejection, but prior to the date of filing a brief, will not be entered because
   (a) ☐ They raise new issues that would require further consideration and/or search (see NOTE below);
   (b) ☐ They raise the issue of new matter (see NOTE below);
   (c) ☐ They are not deemed to place the application in better form for appeal by materially reducing or simplifying the issues for appeal; and/or
   (d) ☐ They present additional claims without canceling a corresponding number of finally rejected claims.
   NOTE: _____. (See 37 CFR 1.116 and 41.33(a)).

4. ☐ The amendments are not in compliance with 37 CFR 1.121. See attached Notice of Non-Compliant Amendment (PTOL-324).

5. ☐ Applicant's reply has overcome the following rejection(s): _____.

6. ☐ Newly proposed or amended claim(s) _____ would be allowable if submitted in a separate, timely filed amendment canceling the non-allowable claim(s).

7. ☒ For purposes of appeal, the proposed amendment(s): a) ☐ will not be entered, or b) ☒ will be entered and an explanation of how the new or amended claims would be rejected is provided below or appended.
   The status of the claim(s) is (or will be) as follows:
   Claim(s) allowed: _____.
   Claim(s) objected to: _____.
   Claim(s) rejected: *1,3,4,7,19 and 23-27.*
   Claim(s) withdrawn from consideration: _____.

AFFIDAVIT OR OTHER EVIDENCE

8. ☐ The affidavit or other evidence filed after a final action, but before or on the date of filing a Notice of Appeal will not be entered because applicant failed to provide a showing of good and sufficient reasons why the affidavit or other evidence is necessary and was not earlier presented. See 37 CFR 1.116(e).

9. ☐ The affidavit or other evidence filed after the date of filing a Notice of Appeal, but prior to the date of filing a brief, will not be entered because the affidavit or other evidence failed to overcome all rejections under appeal and/or appellant fails to provide a showing a good and sufficient reasons why it is necessary and was not earlier presented. See 37 CFR 41.33(d)(1).

10. ☐ The affidavit or other evidence is entered. An explanation of the status of the claims after entry is below or attached.

REQUEST FOR RECONSIDERATION/OTHER

11. ☒ The request for reconsideration has been considered but does NOT place the application in condition for allowance because: See Continuation Sheet.

12. ☐ Note the attached Information *Disclosure Statement*(s). (PTO/SB/08) Paper No(s). _____

13. ☐ Other: _____.

/A R Salimi/
Primary Examiner, Art Unit 1648

/M. G. H./
Examiner, Art Unit 1648

Continuation of 11. does NOT place the application in conditon for allowance because: Applicant argues that there are two distinct embodiements of the invention (one to increase the other decrease T-cell mediated resonses), that there is no enablement differentiation between large molecules and small in the MPEP or case law and that antibodies bind with exquisite specificity, that Pereira et al is supplied again and shows in vivo, and finally that enablement bear reasonable scope to the claims..
The two embodiements both comprise administering an antibody that binds SEQ ID# 2. While the specification provides written describltion for the recited claims and has in vitro experiments to show AZN-D1,2 inhibit T cell response 75% to DC-SIGN (Example 7), there is no teaching of the amount or type of reduced immune response or what the significance is in vivo or what that level of reducing produces. The examiner does not doubt the specificity of antibody binding but the claims are drawn to a method that requires more than mere antibody binding. The Pereira et al. reference is now seen by the examiner and noting the present arguments and those in the last response (page 10 of 6/8/09), the paper shows efficient targeting using the antibody AZN-D1 and that in the paper and specifically in the conclusion, it refers to using the antibody used in antigen targeting of DC-SIGN.
The rejection is maintained.

EVIDENCE APPENDIX D
COPY OF INGULLI ET AL.

In Vivo Detection of Dendritic Cell Antigen Presentation to CD4+ T Cells

By Elizabeth Ingulli,* Anna Mondino,‡§ Alexander Khoruts,‡§ and Marc K. Jenkins‡§

*From the *Department of Pediatrics, ‡Department of Microbiology, and the §Center for Immunology, University of Minnesota Medical School, Minneapolis, Minnesota 55455*

Summary

Although lymphoid dendritic cells (DC) are thought to play an essential role in T cell activation, the initial physical interaction between antigen-bearing DC and antigen-specific T cells has never been directly observed in vivo under conditions where the specificity of the responding T cells for the relevant antigen could be unambiguously assessed. We used confocal microscopy to track the in vivo location of fluorescent dye-labeled DC and naive TCR transgenic CD4+ T cells specific for an OVA peptide–I-A^d complex after adoptive transfer into syngeneic recipients. DC that were not exposed to the OVA peptide, homed to the paracortical regions of the lymph nodes but did not interact with the OVA peptide-specific T cells. In contrast, the OVA pepude-specific T cells formed large clusters around paracortical DC that were pulsed in vitro with the OVA peptide before injection. Interactions were also observed between paracortical DC of the recipient and OVA peptide-specific T cells after administration of intact OVA. Injection of OVA peptide-pulsed DC caused the specific T cells to produce IL-2 in vivo, proliferate, and differentiate into effector cells capable of causing a delayed-type hypersensitivity reaction. Surprisingly, by 48 h after injection, OVA peptide-pulsed, but not unpulsed DC disappeared from the lymph nodes of mice that contained the transferred TCR transgenic population. These results demonstrate that antigen-bearing DC directly interact with naive antigen-specific T cells within the T cell–rich regions of lymph nodes. This interaction results in T cell activation and disappearance of the DC.

In vitro and in vivo studies have shown that bone marrow–derived dendritic cells (DC)[1] are the most effective APCs at activating naive CD4+ T cells (1). This efficiency is thought to be related to the fact that DC express high levels of class II MHC, adhesion, and costimulatory molecules, which play essential roles in T cell activation (1). In addition, DC are the most abundant class II MHC–expressing APC in the T cell–rich areas of secondary lymphoid tissues, and are thus well-positioned to interact with naive T cells.

Although in vitro experiments strongly indicate that DC are the initiating APC for T cell responses in vivo, suggestive evidence supporting this idea has only recently been reported (2, 3). Proliferating T cells have been observed in contact with the DC of the T cell areas of lymphoid tissue after injection of superantigens (2) or allogeneic cells (3). However, because methodologies did not exist for in situ detection of the few T cells specific for any given peptide–MHC complex in unimmunized individuals, it has not been possible to definitively demonstrate interactions between naive, peptide antigen-specific T cells and antigen-bearing dendritic cells in vivo. To overcome this difficulty, we previously developed (5) an adoptive transfer system in which a small but detectable number of naive DO11.10 TCR transgenic CD4+ cells are injected intravenously into normal syngeneic BALB/c recipients. The majority of the CD4+ T cells in the DO11.10 TCR transgenic mice (6) are specific for a chicken OVA peptide 323–339/I-A^d class II MHC complex (7), and can be detected with the KJ1-26 mAb (8), which is uniquely specific for the DO11.10 clonotypic TCR. We used this system here, to characterize interactions between peptide–MHC-bearing DC and naive antigen-specific CD4+ T cells during in vivo immune responses.

Materials and Methods

Animals. DO11.10 BALB/c mice were produced by backcrossing the original DO11.10 TCR-transgenic mice (kindly provided by Dr. Dennis Loh) with BALB/c ($H\text{-}2^d$) mice (pur-

[1] *Abbreviations used in this paper:* CMFDA, 5-chloromethylfluorescein diacetate; CMTMR, 5-(and -6)-((4-chloromethyl) benzoyl) amino) tetramethylrhodamine; DC, dendritic cell; DTH, delayed-type hypersensitivity; SA, streptavidin.

chased from the National Cancer Institute, Frederick, MD) for >10 generations. DO11.10 BALB/c SCID mice were produced by back-crossing DO11.10 BALB/c mice with BALB/c SCID mice (purchased from the National Cancer Institute, Frederick, MD) for two generations and selecting for offspring that contained DO11.10 T cells and no other lymphocytes (as assessed by flow cytometric analysis of blood cells stained with KJ1-26 and anti-B220 mAbs). All DO11.10 BALB/c and BALB/c SCID mice were bred and housed under specific pathogen-free conditions. 6–8-wk-old, sex-matched mice were used for all experiments. Mice were cared for in accordance with University of Minnesota and NIH guidelines.

Purification and Labeling of CD4⁺ T Cells. Lymph node cells from DO11.10 or BALB/c donor mice were passed over an anti-CD8 and anti-immunoglobulin column (Biotex, Edmonton, Alberta) to enrich for CD4⁺ T cells. Lymph node cells from DO11.10 SCID mice were passed over a G10 column to eliminate macrophages. The T cells were labeled with the green fluorescent dye, 5-(chloromethyl)fluorescein diacetate (CMFDA), according to the manufacturer's protocol (Molecular Probes, Eugene, OR) and 2.5×10^6 labeled cells were injected intravenously into syngeneic BALB/c mice.

Isolation and Labeling of DC. Splenic DC were enriched according to the method of Steinman and coworkers (9). In brief, spleen fragments were subjected to mild collagenase digestion at 37°C for 60 min to release DC. Low density cells were selected by centrifugation on a 35% bovine serum albumin gradient (Sigma Chem. Co., St. Louis, MO), cultured in plastic dishes for 1–2 h after which the nonadherent cells were washed away. The adherent cells were cultured overnight with 100 μg/ml of OVA peptide 323-339 (referred to hereafter as OVA peptide) or medium. The DC, which detached from the plates during the incubation period, were collected and labeled with the red fluorescent dye, 5-(and -6)-[((4-chloromethyl) benzoyl) amino) tetramethylrhodamine (CMTMR), according to the manufacturer's protocol (Molecular Probes). DC were injected subcutaneously (0.5×10^6 in 30–50 μl of PBS) into the hind foot pads of recipient mice the day after the DO11.10 T cell transfer. Splenic DC were used instead of the DC that can be obtained from cytokine-stimulated bone marrow cultures, to minimize potential presentation of FCS serum proteins that could lead to background detection of FCS-specific T cells (10).

Flow Cytometry. DC purity was confirmed by flow cytometry as described by Levin et al. (11). In brief, purified DC (10^6) were incubated with FITC-labeled anti-FcR mAb 2.4G2 (PharMingen, San Diego, CA), FITC-labeled anti-B220 mAb RA3-6B2 (PharMingen), biotin-labeled anti-class II MHC mAb M5/114, and PE-labeled streptavidin (SA) (Caltag, South San Francisco, CA) sequentially on ice for 15–20 min, with washes between steps. 10,000 events were collected on a Becton Dickinson FACScan® (Mountain View, CA) flow cytometer and analyzed using Lysis II software. DC were identified as class II MHC⁺, Fc receptor⁻, B220⁻ cells; and ~85% of the cells were DC by these criteria. To obtain the percentage of CD4⁺, KJ1-26⁺ cells, popliteal lymph node cells (10^6) were stained with PE-labeled anti-CD4 mAb H129.19 (PharMingen) and biotin-labeled KJ1-26 mAb followed by SA-FITC (Caltag). After washing, 10,000 events were collected and analyzed. For intracellular IL-2 staining, popliteal lymph node cells ($1\text{-}5 \times 10^6$) were stained with CyChrome-labeled anti-CD4 mAb RM4-5 (PharMingen) and biotin-labeled KJ1-26 mAbs followed by SA-FITC. Cells were then fixed in 2% formaldehyde, and permeabilized with 0.5% saponin, as previously described (12, 13). A PE-labeled anti-IL-2 mAb S4B6 (PharMingen) or isotype control mAb R35-95 (PharMingen) was then added to detect cytosolic IL-2. After washing, 1,000–3,000 CD4⁺, KJ1-26⁺ or CD4⁺, KJ1-26⁻ events were collected and analyzed.

Immunofluorescent Microscopy. Draining popliteal lymph nodes were harvested from sacrificed mice at various times after the DC injections. The lymph nodes were frozen in liquid nitrogen. Cryostat-cut tissue sections (24 μm) were fixed in 4% paraformaldehyde and washed in PBS. In some experiments, lymph node sections (4 μm) were fixed in acetone, blocked with anti-FcR mAb 2.4G2 and stained sequentially with biotin-labeled N418 mAb (anti-CD11c), biotin-labeled goat anti–hamster IgG (Caltag), followed by Cy 3-labeled SA. Confocal microscopy and image analyses were performed as previously described by Brelje et al. (14). In brief, sections were analyzed using a confocal microscope equipped with a krypton/argon laser (MRC-1000; Bio-Rad Life Science Group, Hercules, CA). Separate green and red images were collected for each section analyzed. Final image processing was performed using the Confocal Assistant program (Minneapolis, MN) and Adobe Photoshop (Mountain View, CA). The area (mm^2) of lymph node scanned was measured using MetaMorph software (West Chester, PA). Adjacent tissue sections (4 μm) were stained with biotin-labeled anti-CD4 mAb RM4-5 (PharMingen) and SA-FITC (Caltag) or biotin-labeled anti-B220 mAb RA3-6B2 (PharMingen) and SA-tetramethylrhodamine (Molecular Probes) to identify the paracortical (T cell–rich) and follicular (B cell–rich) regions of the lymph nodes by conventional immunofluorescent microscopy.

Injections. In some experiments, mice were injected subcutaneously in the hind foot pad with a chemical conjugate of OVA and hen egg lysozyme, which was designed for use in T cell/B cell collaboration experiments. This conjugate, and native OVA stimulate DO11.10 T cells identically in vivo (data not shown).

Delayed-type Hypersensitivity Response. 7 d after the initial DC injections, mice were rechallenged with an intradermal injection of soluble OVA (10 μg) in the ears. Ear thickness was measured at the time of injection (baseline) and 24 h later by an individual who was unaware of the experimental design. The degree of response was calculated as the difference between the two measurements.

Results

In Vivo Analysis of Interactions between Antigen-pulsed DC and Antigen-specific CD4⁺ T Cells. Using immunohistochemical detection with the KJ1-26 mAb, we previously showed that DO11.10 T cells were present in the T cell–rich paracortical regions of all lymph nodes within 24 h of intravenous injection (5). Similarly, intravenously injected, CMFDA-labeled DO11.10 T cells were found in the paracortical regions of the lymph nodes (Fig. 1) 24 h after injection, indicating that the dye labeling process did not affect their trafficking ability. In the absence of antigen, the CMFDA-labeled DO11.10 T cells could be detected via their green fluorescence in the paracortical regions for at least 72 h (data not shown). CMTMR-labeled, unpulsed DC were first detected in the draining lymph nodes 8 h after subcutaneous injection, accumulated to a maximal level by 24 h, and declined slightly by 48 h (Figs. 2 *B* and 3 *A*). By 24 h, the unpulsed DC were found almost exclusively in the paracortical regions of the lymph nodes (Fig. 1 *B*). Despite the paracortical colocalization of the labeled T cells and un-

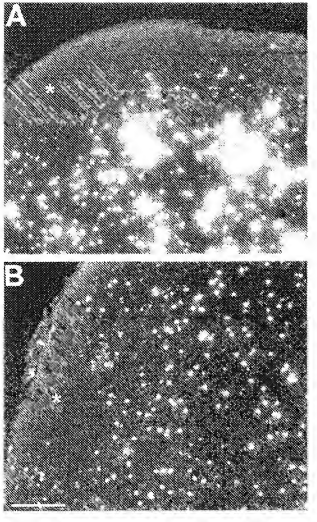

Figure 1. Visualization of OVA peptide–MHC-bearing DC and $CD4^+$ T cell interactions in situ. CMFDA-labeled DO11.10 T cells (*green*) and CMTMR-labeled DC (*red*) were purified, dye-labeled and injected into recipient mice as described in Materials and Methods. Draining popliteal lymph nodes were harvested 24 h after DC injections. Tissue was processed and analyzed by confocal microscopy as described in Materials and Methods. The full-width half peak resolution of the sampling volume was 4.5–5.0 μm; in other words, the optical thickness of each image is 4.5–5 μm. Images were taken from lymph nodes of mice injected with (*A*) OVA peptide-pulsed DC or (*B*) unpulsed DC. Follicular regions, defined on adjacent sections as areas rich in B220$^+$ cells, are indicated (*). Bar, 150 μm.

pulsed DC, few if any interactions between the cells were observed throughout the 48-h observation period (Figs. 1 *B*, 2 *B*, and 3 *B*).

A very different pattern was observed after injection of OVA peptide-pulsed DC. The rate of entry of OVA peptide-pulsed DC into the lymph nodes over the first 24 h was similar to that observed for unpulsed DC (Figs. 2 *A* and 3 *A*). However, at each time point, clusters of DO11.10 T cells (≥2 cells) were observed surrounding the OVA peptide-pulsed DC (Figs. 1 *A*, 2 *A*, and 3 *B*). At 24 h, the time of maximal DC accumulation, ~70% of the OVA peptide-pulsed DC were surrounded by many DO11.10 T cells. In addition, at this magnification, areas of yellow color appeared in the clusters reflecting overlap between the red and green images and close contact between the T cells and DC. The specificity of cluster formation was indicated by the finding that clusters were not observed in mice that received CMFDA-labeled polyclonal BALB/c $CD4^+$ T cells and were injected 24 h previously with CMTMR-labeled OVA peptide-pulsed DC (Fig. 3 *B*).

Although the high degree of coincidence between OVA peptide-pulsed DC and DO11.10 T cell clusters at 24 h made it likely that the clusters represented bona fide interactions between the two cell types, the ~5-μm-thickness of the optical sections made it formally possible that the dendritic cells were not centrally located within the clusters, but rather were situated immediately above or below the clusters. Serial optical sectioning through individual cluster-associated, OVA peptide-pulsed dendritic cells was performed at high power (optical section thickness = 0.6

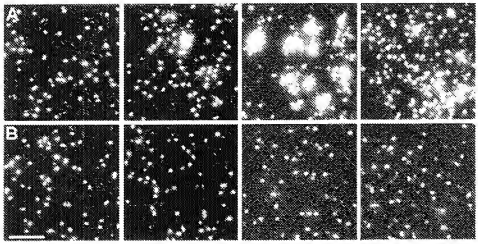

Figure 2. In vivo clustering of OVA peptide–MHC-bearing DC and DO11.10 T cells. DO11.10 T cells (*green*) and DC (*red*) were purified, dye-labeled and injected into recipient mice as described in Materials and Methods. Draining popliteal lymph nodes were harvested 4, 8, 24, and 48 h after DC injections. Tissue was processed and analyzed by confocal microscopy as described in Materials and Methods. The optical thickness of each image is 4.5–5 μm. Images were taken from paracortical regions of lymph nodes of mice injected with (*A*) OVA peptide-pulsed DC or (*B*) unpulsed DC. Bar, 190 μm.

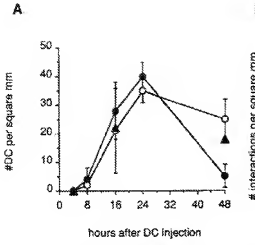

Figure 3. Kinetics of DC appearance and cluster formation in draining lymph nodes. CMFDA-labeled DO11.10 (*nicles*) or polyclonal BALB/c (*triangles*) $CD4^+$ T cells were injected intravenously into BALB/c recipient mice the day before CMTMR-labeled OVA peptide-pulsed (*closed symbols*) or unpulsed (*open symbols*) DC were injected subcutaneously into the hind foot pads. Draining popliteal lymph nodes were harvested at the indicated times after DC injection and analyzed with two-color confocal immunofluorescent microscopy as described in Materials and Methods. One image was collected per 24-μm section to ensure that individual fluorescent cells were only counted once. An interaction was defined as two or more green T cells overlapping a red DC such that a yellow area was produced. The number of DC (*A*) and DC engaged in T cell interactions (*B*) were quantified per unit area (mm^2) of lymph node. The results represent the mean values ± SD of 2–3 mice/group (except for the results from the polyclonal BALB/c T cell group, which came from a single animal) derived from a single experiment. Similar values were obtained in two other independent experiments.

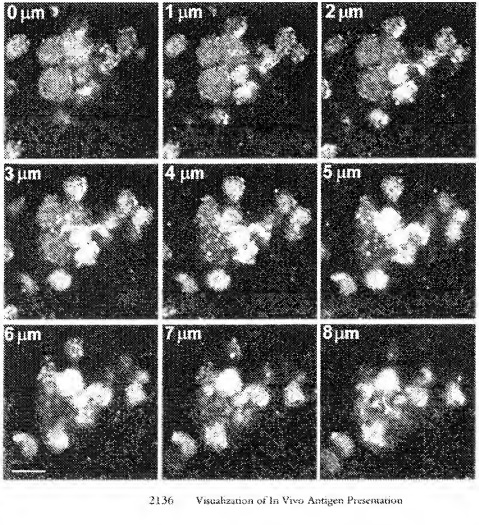

Figure 4. Detailed analysis of a DC–T cell cluster. A series of optical sections was taken at 1-μm intervals through a dendritic cell that appeared to be surrounded by T cells 24 h after the injection of OVA peptide-pulsed DC. At this magnification, the optical thickness of each image is ~0.6 μm. Bar, 10 μm.

μm) to assess this possibility. As shown in Fig. 4, interactions between the dendritic cell and several DO11.10 T cells were observed in each serial plane of focus through the dendritic cell body. Similar results were observed for all clusters examined in this way (n = 5). These results demonstrate that OVA peptide-pulsed dendritic cells are centrally located within the clusters at the 24-h time point, and are simultaneously interacting with many DO11.10 T cells.

48 h after injection, essentially all of the OVA peptide-pulsed DC were surrounded by DO11.10 T cells (Fig. 2 *A*). However, the number of DO11.10 T cells present in the clusters was reduced and the number of OVA peptide-pulsed DC present was lower than was observed in recipients of unpulsed DC at this time (Fig. 3 *A*). The disappearance of OVA peptide-pulsed DC at 48 h appeared to be a function of the presence of DO11.10 T cells and the ability to induce cluster formation, because OVA peptide-pulsed DC persisted to the same degree as unpulsed DC when injected into mice that previously received CMFDA-labeled polyclonal BALB/c T cells (Fig. 3 *A*).

Approximately 75% of the $CD4^+$ cells purified from the DO11.10 mice express the DO11.10 TCR-α and TCR-β chains (6, 15). Because of incomplete allelic exclusion, the remaining 25% express the DO11.10 TCR-β chain with an endogenous TCR-α chain, and are specific for antigens other than OVA (15). DO11.10 mice were backcrossed with SCID mice, which produce endogenous TCR-α chains only at very low levels, to exclude the possibility that endogenous TCR-α chain–expressing T cells were interacting with the DC. Flow cytometric analysis of lymph node cells from DO11.10 SCID mice confirmed that essentially all of the $CD4^+$ T cells in these mice also stained with the KJ1-26 mAb (data not shown). CMFDA-labeled DO11.10 SCID T cells clustered around 36 ± 3% of the CMTMR-labeled, OVA peptide-pulsed DC and 0 ± 0% of the CMTMR-labeled unpulsed DC, 24 h after DC injection.

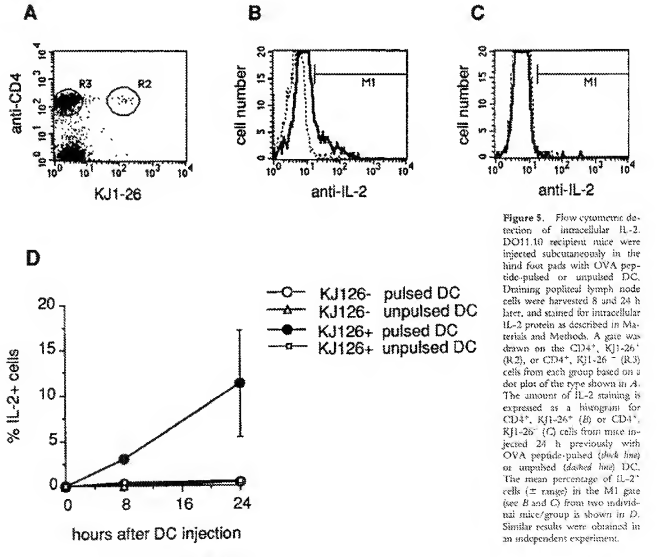

Figure 5. Flow cytometric detection of intracellular IL-2. DO11.10 recipient mice were injected subcutaneously in the hind foot pads with OVA peptide-pulsed or unpulsed DC. Draining popliteal lymph node cells were harvested 8 and 24 h later, and stained for intracellular IL-2 protein as described in Materials and Methods. A gate was drawn on the $CD4^+$, KJ1-26$^+$ (R2), or $CD4^+$, KJ1-26$^-$ (R3) cells from each group based on a dot plot of the type shown in *A*. The amount of IL-2 staining is expressed as a histogram for $CD4^+$, KJ1-26$^+$ (*B*), or $CD4^+$, KJ1-26$^-$ (*C*) cells from mice injected 24 h previously with OVA peptide-pulsed (*thick line*) or unpulsed (*dashed line*) DC. The mean percentage of IL-2$^+$ cells (± range) in the M1 gate (see *B* and *C*) from two individual mice/group is shown in *D*. Similar results were obtained in an independent experiment.

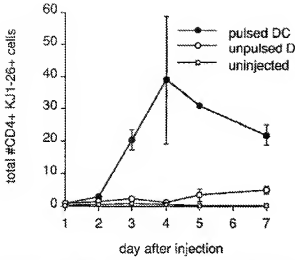

Figure 6. Kinetics of clonal expansion induced by injection of OVA peptide-pulsed or unpulsed DC. BALB/c recipients of DO11.10 T cells were injected subcutaneously in the hind foot pad with 0.5×10^6 OVA peptide-pulsed DC (*closed circles*), 0.5×10^6 unpulsed DC (*open circles*), or nothing (*open squares*). The draining popliteal lymph nodes were harvested at various time points after injection. Flow cytometric analysis was performed on 10,000 lymph node cells from each group after staining to obtain the percentage of $CD4^+$, $KJ1\text{-}26^+$ cells present at each time point. The total number of $CD4^+$, $KJ1\text{-}26^+$ cells was calculated by multiplying the percentage of $CD4^+$, $KJ1\text{-}26^+$ cells by the total number of lymph node cells obtained from a viable cell count. The results represent the mean values ± range of two mice from a single experiment. Similar values were obtained from three other independent experiments.

In this same experiment, we found that CMFDA-labeled DO11.10 SCID T cells also failed (0 ± 0%) to cluster around CMTMR-labeled unpulsed DC in mice that also received unlabeled OVA peptide-pulsed DC at the same time. Together these results demonstrated that $CD4^+$ T cells expressing the DO11.10 TCR were responsible for cluster formation and that cluster formation required that the OVA peptide-pulsed DC present the relevant peptide–MHC complex. Furthermore, because the DO11.10 SCID T cells uniformly expressed a naive surface phenotype ($CD45RB^{high}$, $L\text{-selectin}^{high}$) at the time of adoptive transfer (15, data not shown), these results also demonstrate that naive antigen-specific T cells interact with peptide–MHC-bearing DC in vivo.

The physical interactions between the DO11.10 T cells and OVA peptide-pulsed DC correlated with activation of the T cells. Intracellular staining with anti–IL-2 antibody showed that a significant fraction of the DO11.10 T cells present in recipients injected with OVA peptide-pulsed DC at the time of maximal cluster formation (24 h) were producing the growth factor IL-2 (Fig. 5, *B* and *D*). The specificity of this response was demonstrated by the finding that IL-2 production was not detected in DO11.10 cells from mice injected with unpulsed DC (Fig. 5, *B* and *D*) or in the $CD4^+$, $KJ1\text{-}26^-$ T cells (recipient T cells) present in mice injected with OVA peptide-pulsed DC (Fig. 5, *C* and *D*).

48 h after injection of OVA peptide-pulsed DC about one-third of the CMFDA-labeled DO11.10 T cells were dull green (Fig. 2 *A*), perhaps because of dilution of the dye as a consequence of cell division. By 72 h, the number of DO11.10 T cells in mice injected with OVA peptide-pulsed DC, but not unpulsed DC increased dramatically in the draining lymph nodes, and this increase continued to a peak at 96 h (Fig. 6). Normal mice or recipients of DO11.10 T cells that had been injected with DC 7 d earlier were rechallenged with soluble OVA subcutaneously in the ear to determine if T cells capable of causing an OVA-specific DTH response had been induced. DO11.10 recipient mice that had been previously injected with OVA peptide-pulsed DC, but not unpulsed DC mounted a strong DTH reaction that surpassed that of similarly primed BALB/c mice that did not receive DO11.10 T cells (Fig. 7), suggesting that the DO11.10 T cells participated in the delayed-type hypersensitivity (DTH) reaction in the former situation.

In Vivo Analysis of Interactions Between Resident Lymph Node DC and Antigen-specific $CD4^+$ T Cells after Injection of Soluble Antigen. Finally, we determined whether the unlabeled endogenous DC of the recipient could form clusters with CMFDA-labeled DO11.10 T cells in vivo after injection of a soluble form of intact OVA to ensure that cluster formation was a property of DC that had not been through the purification and labeling process. As shown in Fig. 8, in the absence of OVA, CMFDA-labeled DO11.10 SCID T cells were not clustered and were rarely found interacting with endogenous paracortical DC (Fig. 8 *B*). In contrast, 24 h after injection of OVA, CMFDA-labeled DO11.10 T cells formed small clusters, many of which showed evidence (*yellow*) of interactions with endogenous paracortical DC of the recipient (Fig. 8 *A*).

Discussion

The ability to physically monitor the anatomic location of naive antigen-specific T cells and DC allowed us to reach several conclusions about antigen presentation in vivo. Because T cell/DC clusters were only observed when a high frequency of OVA peptide-specific T cells and OVA peptide–MHC-bearing DC were present at the same time is strong evidence that the clusters represent antigen presentation events. This conclusion is further supported by the findings that cluster formation coincided with IL-2 production by the antigen-specific T cells, and was followed by proliferation and differentiation of the T cells into DTH effector cells. Our results are consistent with those of others who showed that clusters of proliferating T cells are found in proximity to T cell zone DC after injection of superantigens (2) or allogeneic cells (3). The capacity of individual OVA peptide-pulsed DC to simultaneously interact with many antigen-specific T cells in vivo is reminiscent of the in vitro studies of Steinman and coworkers (16–19), and provides a possible explanation for the ability of small numbers of tumor peptide-pulsed DC to induce cancer im-

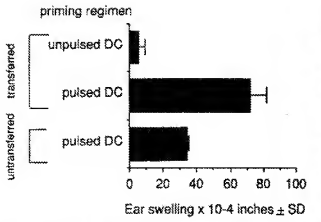

Figure 7. DTH response. DO11.10 recipient mice were injected subcutaneously in the hind foot pad with 0.5×10^6 OVA peptide-pulsed or unpulsed DC. In addition, normal BALB/c mice were injected with 0.5×10^6 OVA peptide-pulsed DC. 7 d after the initial injection, all mice were rechallenged with an intradermal injection of soluble intact OVA (10 μg) in the ears. The results represent the mean ear swelling values ± SD of 2–4 ears/group derived from a single experiment. Similar values were obtained from three other independent experiments.

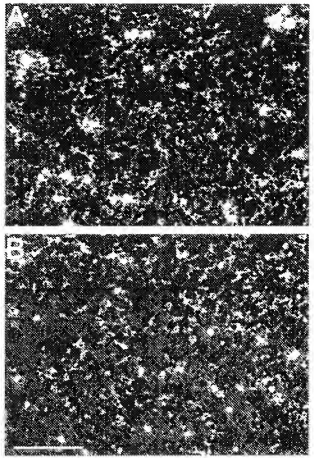

Figure 8. Endogenous DC cluster DO11.10 T cells in the presence of antigen. DO11.10 SCID T cells were purified, CMFDA-labeled (*green*), and injected into recipient mice as described in Materials and Methods. The next day recipient mice were injected subcutaneously with 45 μg of OVA–hen egg lysozyme conjugate (*A*) or nothing (*B*). 24 h later, draining popliteal lymph nodes were harvested, sectioned, fixed in acetone, and stained sequentially with biotin-labeled DC-specific mAb N418, biotin-labeled goat anti–hamster IgG and Cy 3–labeled SA to detect endogenous paracortical DC (*red*). Tissue was analyzed by confocal microscopy as described in Materials and Methods. The optical thickness of each image is 2–3 μm. Images shown were from paracortical regions of the lymph nodes. Bar, 100 μm.

munity (20, 21). It should also be noted that DO11.10 T cells formed clusters around endogenous DC following soluble OVA injection indicating that indeed DC play an important role in antigen presentation in vivo. In this situation, however, DC^{+} T cell clusters are smaller probably because all of the DC have access to antigen and are thus in competition with each other for antigen presentation to the DO11.10 T cells.

The finding that very few DO11.10 T cells were found in proximity to unpulsed DC despite colocalization to the lymph node paracortex indicates that interactions between antigen-specific $CD4^{+}$ T cells and DC in the absence of the relevant antigen are very transient. It is possible that these transient interactions are stabilized if the DC display the appropriate peptide–MHC complexes and activate the interacting T cells to upregulate the activity of their adhesion molecules (22). The subsequent stable binding between antigen-specific T cells and antigen-presenting DC would allow sustained TCR signaling, which has been shown to be required for T cell commitment to lymphokine production (23). An alternative possibility is that naive T cells are first activated by an APC from the recipient before binding to the OVA peptide-pulsed, labeled DC. This is unlikely because it would require transfer of OVA peptide from the labeled DC to recipient APCs. This does not appear to be the case because injection of OVA peptide-pulsed, unlabeled DC together with unpulsed, labeled DC into recipients containing labeled DO11.10 T cells did not result in cluster formation between the labeled populations as would be expected if peptide transfer occurred.

It was surprising to find that OVA peptide-pulsed, CMTMR-labeled DC rapidly disappeared from the lymph nodes after the time of maximal interaction with DO11.10 T cells. It is possible that activation caused the labeled DC to metabolize the dye and become undetectable. CD40 signaling has been shown to stimulate cytokine production and costimulatory molecule expression in DC, and the activated DO11.10 T cells present in the clusters would be expected to express CD40 ligand (24). However, if dye metabolism due to DC activation was the only explanation, then many DO11.10 T cell clusters lacking a labeled DC should have been observed. This was not the case; many fewer clusters were present at 48 h but most contained a labeled DC. Thus, a more likely explanation is that the DC physically disappear because they migrate out of the lymph node or are killed by the responding T cells. $CD4^{+}$ T cell killing of the cognate APC has been described in several cases (25, 26). In addition, a recent study by De Smedt et al.

(27) showed that LPS also induces the activation and then disappearance of DC in the spleen. Therefore, DC activation by inflammatory cytokines or cognate interactions with antigen-specific T cells may eventually lead to elimination of the DC, allowing any interacting T cells to disengage. By escaping from antigen-presenting DC, activated T cells would be free to proliferate, interact with other APCs such as antigen-specific B cells within the follicles, and eventually to migrate out of the lymph node to non-lymphoid sites of inflammation. Transient elimination of antigen-presenting DC would also provide a mechanism for terminating T cell responses, that could do damage if allowed to go on unchecked.

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References

1. Steinman, R.M. 1991. The dendritic cell system and its role in immunogenicity. *Annu. Rev. Immunol.* 9:271–296.
2. Lather, S.A., A. Gulbranson-Judge, H. Acha-Orbea, and I. MacLennan. 1997. Viral superantigen drives extrafollicular and follicular B cell differentiation leading to virus-specific antibody production. *J. Exp. Med.* 185:551–562.
3. Kudo S., K. Matsuno, T. Ezaki, and M. Ogawa. 1997. A novel migration pathway for rat dendritic cells from the blood: hepatic sinusoids-lymph translocation. *J. Exp. Med.* 185:777–784.
4. Tse, H.Y., R.H. Schwartz, and W.E. Paul. 1980. Cell-cell interactions in the T cell proliferative response. *J. Immunol.* 125:401–500.
5. Kearney, E.R., K.A. Pape, D.Y. Loh, and M.K. Jenkins. 1994. Visualization of peptide-specific T cell immunity and peripheral tolerance induction in vivo. *Immunity.* 1:327–339.
6. Murphy, K.M., A.B. Heimberger, and D.Y. Loh. 1990. Induction by antigen of intrathymic apoptosis of $CD4^+CD8^+$ TCR^{lo} thymocytes in vivo. *Science (Wash. DC).* 250:1720–1723.
7. Shimonkevitz, R., S. Colon, J.W. Kappler, P. Marrack, and H.M. Grey. 1984. Antigen recognition by H-2-restricted T cells. II. A tryptic ovalbumin peptide that substitutes for processed antigen. *J. Immunol.* 133:2067–2074.
8. Haskins, K., R. Kubo, J. White, M. Pigeon, J. Kappler, and P. Marrack. 1983. The MHC-restricted antigen receptor on T cells. I. Isolation of a monoclonal antibody. *J. Exp. Med.* 157:1149–1169.
9. Swiggard, W.J., R.M. Nonaes, M.D. Witmer-Pack, R.M. Steinman, and K. Inaba. 1991. Enrichment of dendritic cells by plastic adherence and EA rosetting. *In* Current Protocols in Immunology. J.E. Coligan, A.M. Kruisbeek, D.H. Margulies, E.M. Shevach, and W. Strober, editors. John Wiley and Sons, New York. 3.7.1–3.7.11.
10. Inaba, K., M. Inaba, N. Romani, H. Aya, M. Deguchi, S. Ikehara, S. Muramatsu, and R.M. Steinman. 1992. Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor. *J. Exp. Med.* 176:1693–1702.
11. Levin, D., S. Constant, T. Pasqualini, R. Flavell, and K. Bottomly. 1993. Role of dendritic cells in the priming of $CD4^+$ T lymphocytes to peptide antigen in vivo. *J. Immunol.* 151: 6742–6750.
12. Assenmacher, M., J. Schmitz, and A. Radbruch. 1994. Flow cytometric determination of cytokines in activated murine T helper lymphocytes: expression of interleukin-10 in interferon-γ and interleukin-4-expressing cells. *Eur. J. Immunol.* 24:1097–1101.
13. Openshaw P., E.E. Murphy, N.A. Hosken, V. Maino, K. Davis, K. Murphy, and A. O'Garra. 1995. Heterogeneity of intracellular cytokine synthesis at the single-cell level in polarized T helper 1 and T helper 2 populations. *J. Exp. Med.* 182:1357–1367.
14. Brelje T.C., M.W. Wessendorf, and R.L. Sorenson. 1993. Multi-color laser scanning confocal immunofluorescence microscopy: practical application and limitations. *Methods Cell Biol.* 38:97–181.
15. Lee, W.T., J. Cole-Calkins, and N.E. Street. 1996. Memory T cell development in the absence of specific antigen priming. *J. Immunol.* 157:5300–5307.
16. Flechner, E.R., P.S. Freudenthal, G. Kaplan, and R.M. Steinman. 1988. Antigen-specific T lymphocytes efficiently cluster with dendritic cells in the human primary mixed-leukocyte reaction. *Cell. Immunol.* 111:183–195.
17. Inaba, K., M. Witmer, and R.M. Steinman. 1984. Clustering of dendritic cells, helper T lymphocytes, and histocompatible B cells during primary antibody responses in vitro. *J. Exp. Med.* 160:858–876.
18. Inaba, K., and R.M. Steinman. 1986. Accessory cell–T lymphocyte interactions. *J. Exp. Med.* 163:247–261.
19. Austyn, J.M., D.E. Weinstein, and R.M. Steinman. 1988. Clustering with dendritic cells precedes and is essential for T-cell proliferation in a mitogenesis model. *Immunology.* 63:691–696.
20. Mayordomo J.I., T. Zorina, W.J. Storkus, L. Zitvogel, C. Celluzzi, L.D. Falo, C.J. Melief, S.T. Ildstad, W.M. Kast, A.B. Deleo, and M.T. Lotze. 1995. Bone marrow-derived dendritic cells pulsed with synthetic tumour peptides elicit protective and therapeutic antitumor immunity. *Nat. Med.* 1:

1297–1302.
21. Steinman, R.M. 1996. Dendritic cells and immune-based therapies. *Exp. Hematology.* 24:859–862.
22. Dustin, M.L., and T.A. Springer. 1989. T cell receptor cross-linking transiently stimulates adhesiveness through LFA-1. *Nature (Lond.).* 341:619–624.
23. Valitutti S., M. Dessing, K. Aktories, H. Gallati, and A. Lanzavecchia. 1995. Sustained signaling leading to T cell activation results from prolonged T cell receptor occupancy. Role of T cell actin cytoskeleton. *J. Exp. Med.* 181:577–584.
24. Noelle, R.J. 1996. CD40 and its ligand in host defense. *Immunity.* 4:415–419.
25. Tite, J.P. 1990. Evidence of a role for TNF-alpha in cytolysis by $CD4^+$, class II MHC-restricted cytotoxic T cells. *Immunology.* 71:208–212.
26. Rathmell, J.C., M.P. Cooke, W.Y. Ho, J. Grein, S.E. Townsend, M.M. Davis, and C.C. Goodnow. 1995. CD95 (Fas)-dependent elimination of self-reactive B cells upon interaction with $CD4^+$ T cells. *Nature (Lond.).* 376:181–184.
27. De Smedt, T., B. Pajak, E. Muraille, L. Lespagnard, E. Heinen, P. De Baetselier, J. Urbain, O. Leo, and M. Moser. 1996. Regulation of dendritic cell numbers and maturation by lipopolysaccharide in vivo. *J. Exp. Med.* 184:1413–1424.

EVIDENCE APPENDIX E
COPY OF STEINMAN

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Appendix E

DC-SIGN: A Guide to Some Mysteries of Dendritic Cells

Minireview

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During the last decade, dendritic cells (DCs) have come to be appreciated as critical controllers of the immune response, especially T cell responses. Although T lymphocytes actually mediate resistance to infections, transplants, and even tumors, without proper instruction from DCs, T cells would be severely compromised. DCs convert antigens from foreign cells and infectious microorganisms into short peptides that are bound to membrane proteins of the major histocompatibility complex (MHC). These MHC–peptide complexes are formed intracellularly but are ultimately presented on the plasma membrane where they serve as ligands for antigen-specific T cell receptors (TCR). In addition to TCR ligand formation, DCs carry out many other functions, some to be considered below, which allow them to control immunity at several points.

Despite their importance, the DC can be regarded as the Cinderella of the immune system, for years kept by the hearths of a few laboratories. With added attention, as illustrated by two papers by Geijtenbeek and colleagues in this issue of *Cell*, one can begin to appreciate some of the DC's glamor. Both papers center on DC-SIGN, a new DC-restricted molecule. DC-SIGN in turn qualifies as Cinderella's glass slipper, as it seems to be used by the dashing T cell and the wicked HIV-1 to identify their DC. Yet the slipper, like its wearer, had to be rescued from years of oblivion. None of the new ideas described in the papers by Geijtenbeek et al. were apparent from the genomic sequence of DC-SIGN, first reported in 1992 and since then deposited several times in gene banks. Instead the research had to shift to DCs for the importance of this molecule to be appreciated.

DC-SIGN is a type II membrane protein with an external mannose-binding, C-type lectin domain. It was cloned from a placental library, through its capacity to bind the glycan-rich HIV-1 envelope in the absence of CD4 (Curtis et al., 1992), the classic virus receptor. In the first of these two papers (Geijtenbeek et al., 2000a), the lectin is rediscovered and renamed DC-SIGN, because it is a "DC-specific, ICAM-3 grabbing, nonintegrin." It is proposed that the interaction of DC-SIGN with ICAM-3 establishes the initial contact of the DC with a resting T cell, helping to explain the potency with which DCs initiate T cell immunity. Potency has long been apparent in tissue culture and in experimental animals, and this adjuvant role of DCs has been extended to humans (Dhodapkar and Bhardwaj, 2000). The second paper (Geijtenbeek et al., 2000b) addresses the known capacity of DCs to promote HIV-1 infection in culture. DC-SIGN proves to be a special kind of viral receptor, promoting binding and transmission of HIV-1 to T cells, rather than viral entry into the DC. Therefore, DC-SIGN likely will be pivotal for explaining some important functions of DCs.

The Potency of DC in Initiating Immune Responses from Resting T Cells

The term "potency" indicates that relatively small numbers of DCs, and relatively low doses of an antigen or other T cell stimulus, are sufficient to initiate rapid and strong responses, such as T cell proliferation and lymphokine production. Potency is not simply a matter of more efficient MHC–peptide complex formation, although this too is a newly recognized mechanism used by DCs to control immunity (Inaba et al., 2000). Instead potency is readily observed with stimuli that do *not* require processing, e.g., polyclonal mitogens, microbial superantigens, and transplantation antigens. DCs are also effective when the amount of membrane-bound TCR ligand is vanishingly small, as few as 100–1000 ligands on the entire cell surface (Bhardwaj et al., 1993). For these reasons, the efficacy of DCs has been attributed to special accessory molecules. Many such molecules are found on DCs, e.g., CD48, -54, -58, -80, -86, and the corresponding antibodies can block DC–T cell interactions. However, these membrane proteins are shared with other antigen-presenting cells. DC-SIGN is the first recognized DC-restricted product that helps stimulate resting T cells.

The Figdor lab realized that resting T cells expressed the adhesion molecule ICAM-3. In contrast to what was expected, ICAM-3 did not bind to β_2 integrins on DCs. When they made antibodies to block ICAM-3 binding to DCs, the monoclonals identified a small 44 kDa molecule. Its binding to ICAM-3 was Ca^{2+} dependent and blocked by mannan. The antibodies reacted specifically with DCs. Cloning showed that the ICAM-3-binding molecule was identical to the previously defined HIV-1 envelope–binding lectin (Curtis et al., 1992). In functional tests, the renamed DC-SIGN contributed to transient DC–T cell clustering and responses to transplantation antigens.

Geijtenbeek et al. (2000a) propose that DC-SIGN mediates the known loose adhesion that takes place between DCs and T cells in the apparent absence of foreign antigen (Figure 1). Such adhesion seems necessary, because MHC–peptide ligands are membrane bound, typically scarce (10–1000 copies/cell), and need to be recognized by the TCR, another membrane molecule. DC-SIGN-mediated adhesion provides an opportunity for the TCR to scan the DC surface to identify these small amounts of TCR ligand, which then activate the resting T cell. Subsequently T cells respond vigorously to antigens presented by other cells, but by then, the activated T cells are replete with their own functional adhesion molecules.

Further experiments will decipher this proposed mechanism of action and dissect DC-SIGN function in vivo. Mice deleted of the gene for DC-SIGN will be valuable, assuming there are no additional homologs. Nevertheless, it is impressive at this early stage of research to recall that human cells have been used to uncover most of the important accessory membrane proteins

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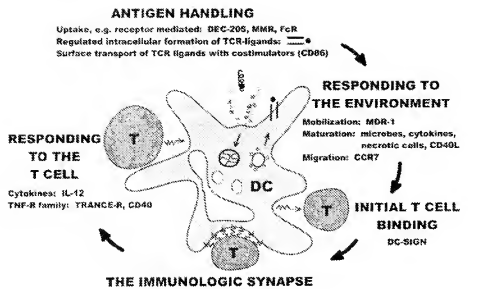

Figure 1. A Proposed Sequence of Action for the Initiation of Immunity by DCs

that control T cell function, not just DC-SIGN and ICAM-3. These include CD58 (LFA-3) for CD2; CD54 (ICAM-1) for CD11a (LFA-1); CD40L (gp39) for CD40; CD80 (B7-1) and CD86 (B7-2) for CD28 and CD154 (CTLA-4) (Figure 1).

Geijtenbeek et al. (2000a) detected DC-SIGN on DCs that were not mature or terminally differentiated, and expression of DC-SIGN did not increase when the DCs matured. This raises the possibility that DC-SIGN is mainly needed by developing DCs, not yet expressing optimal levels of MHC–peptide and accessory molecules like CD86, to contact ICAM-3 on resting T cells and then mature with help from the responding T cells. Conceivably, DC-SIGN could be involved in T cell responses other than classical immunity, such as the induction of tolerance and immune regulation.

Although DC-SIGN could be a long-sought, DC-unique molecule for T cell adhesion, and thereby help to explain the potency of DCs, there has been progress on another hypothesis. DCs are held to have other unique products to enhance signaling together with the TCR, i.e., costimulation. While DCs are remarkable in this regard, to date costimulation is not known to involve a DC-specific product. Instead, the potent costimulator, CD86, is abundant on DCs relative to other antigen-presenting cells. More remarkably, during the vesicular transport of newly formed MHC–peptide complexes in developing DCs, the complexes move together with CD86. Upon arrival at the DC surface, MHC–peptide and CD86 are deposited as stable clusters (Inaba et al., 2000). Therefore DCs are designed to set up the "immunological synapse."

The concept of a synapse, a term first coined by William Paul, proposes a central contact zone in which the APC and T cell membranes are only 134 Å apart (Davis and van der Merwe, 1996; Shaw and Dustin, 1997). The zone contains multiple copies of molecular couples that span this distance, e.g., CD48 or -58 plus CD2, CD80 or -86 plus CD28, and importantly, MHC–peptide plus TCR (Figure 1). This assembly of supramolecular aggregates may facilitate costimulation and sustain the low affinity interaction between TCR ligands and the TCR. Direct experiments on synapses have so far used activated T cells. DCs might allow the concept to be pursued in naive T cells. In sum, more precise mechanisms are beginning to explain DC function: DC-SIGN for T cell adhesion, and preformed aggregates of MHC–peptide and membrane accessories for costimulation.

We have only been discussing the basis for DC potency in vitro. In vivo, DCs are found in peripheral tissues, such as the skin and airways but they can migrate to lymphoid tissues. There, in the T cell areas, the DCs are in full view of the circulating, naive lymphocyte repertoire and can even make chemokines that attract these T cells (Adema et al., 1997). The match of the DC and T cell can then be made via DC-SIGN, allowing the intimate cross-talk between the two cells to begin (Ingulli et al., 1997). Nonetheless, to control immunity, the DC displays other functions prior to the use of DC-SIGN. In the peripheral outposts for antigen entry, DCs are immature requiring a stimulus, such as exposure to microbial products or inflammatory cytokines, for terminal differentiation (Cella et al., 1997). The immature DCs express several receptors for antigen uptake, including other lectins (Figure 1, top). The endocytic system is in turn regulated by a maturation stimulus to efficiently convert antigens to MHC–peptide complexes, in concert with CD86 costimulators as mentioned above (Inaba et al., 2000). DC migration from the periphery to the lymphoid tissues involves mobilization via multidrug resistance receptors (Randolph et al., 1998) and chemotaxis through the CCR7 receptor toward chemokines produced in lymphoid tissues and lymphatic channels (Forster et al., 1999). After DC-SIGN functions, the synapse forms and the resting T cell is activated. Then TNF family members

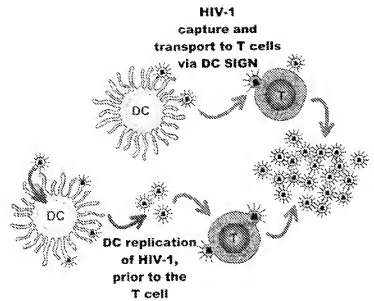

Figure 2. Proposed Pathways for the Transmission of HIV-1 or SIV to Permissive T Cells

The upper, DC-SIGN-dependent pathway does not distinguish M-tropic from T-tropic HIV-1. It may operate, for example, on DCs beneath mucosal epithelial surfaces and is the major pathway in the cells studied by Geijtenbeek et al. (2000b). The lower, infection-dependent pathway may pertain to DCs within mucosal epithelia. Both pathways also could operate in acute and chronic phases of infection in lymphoid tissues.

on the T cell, like CD40L and TRANCE, prolong DC survival and cytokine production (Josien et al., 2000), especially the IL-12 needed for strong T cell–mediated immunity (Figure 1). Therefore DC potency is not due to one surface or secreted molecule. It results from many well-timed and spatially organized specializations.

DCs and the Transmission of HIV-1

The second of the two papers (Geijtenbeek et al., 2000b) reveals a new way for HIV-1 to exploit the DC. The investigators describe a fascinating DC-SIGN–dependent mechanism. This lectin can capture HIV-1 at low external titres. Without allowing viral entry, DC-SIGN retains the attached virus in an infectious state for days and then transmits it to replication-permissive T cells. The in vitro data are fortified with micrographs of tissue sections. DC-SIGN is found on dendritic profiles beneath genital epithelium, a major potential site for HIV-1 transmission, and in the T cell areas, the sites for viral replication especially in acute infection. This pure delivery role for DC-SIGN is consistent with data that, in lymphoid tissues, HIV-1 and SIV mainly replicate in CD4$^+$ T cells, not DCs (Stahl-Hennig et al., 1999).

The new experiments use a standard system to study the involvement of DCs in HIV-1 transmission (Pope et al., 1994). The model is to add HIV-1 to cultured DCs for 1–2 hr, wash, and at varying times, add in T cells and follow the levels and cellular sites of viral replication. A vigorous infection occurs, primarily in T cells. In such a model, antibodies to DC-SIGN exerted a significant but sometimes incomplete block of transmission. When transfected cells were used to pursue the relative roles of DC-SIGN and more classical HIV-1 receptors, DC-SIGN was not an entry receptor and did not influence the entry role of CD4 and CCR5. However, DC-SIGN on one transfectant captured virus, even when present in small amounts, and transmitted the HIV-1 to CD4 and CCR5 on other cells (Figure 2, top). DC-SIGN literally "presents" HIV-1 to T cells, but in a nonprocessed infectious form.

It is possible that other pathogens are also transmitted via DCs and in particular via DC-SIGN. The glycan ligands for this lectin could be present on other viral envelopes, the cell walls of other microbes, or even tumor cells. Also, because DC-SIGN retains its HIV-1 ligand in a native state, this and other lectins could present vaccines to protective B cells, which must react to native antigens. If the vaccine were simultaneously processed and presented to helper T cells, DC-SIGN would even set up an effective DC–T–B "ménage a trois," capable of inducing strong immunity, including mucosal immunity (Fayette et al., 1997).

DC-SIGN is not the only attraction that HIV-1 finds in DCs. The virus can infect certain DCs in culture (Figure 2, bottom, red arrows). HIV-1 is capable of replication in immature DCs, and possibly mature DCs that are interacting with CD40L or T cells (Granelli-Piperno et al., 1999). So while DC-SIGN can transport HIV-1 and enhance infection of T cells, locally or in lymphoid tissues, direct infection could also amplify the amount of virus that DCs deliver. Both pathways (Figure 2) may enhance the overall pathogenesis of HIV-1 infection, although Geijtenbeek et al. (2000b) observe that DC-SIGN in their cells plays the major role in viral replication, especially at low doses of HIV-1.

Importantly, it is well established that M-tropic strains of HIV-1 are preferentially transmitted among humans. One possible explanation is that selection takes place at the level of DC infection. By contrast, DC-SIGN ferries both M- and T-tropic viruses to T cells. Geijtenbeek et al. (2000b) did not detect DC-SIGN on DCs (Langerhans cells) within the genital epithelium, only on DCs beneath the surface. These two subsets of DCs, termed "epidermal or epithelial" and "dermal or subepithelial," represent distinct pathways of differentiation (Caux et al., 1996). Since epidermal, DC-SIGN–negative DCs likely select for M-tropic HIV-1 (Reece et al., 1998), these DCs in vivo may account for the selective transmission of M-tropic HIV-1, which then binds to additional subepithelial, DC-SIGN–positive DCs, greatly amplifying delivery

of virus to T cells locally and eventually lymphoid tissue. Beyond these functions in HIV-1 capture and conveyance, DCs can serve another nefarious role, activating T cells to be permissive for HIV-1 replication (Figure 2, right).

Implications

A new DC-restricted molecule, DC-SIGN, demystifies two of this cell's contrasting functions: stimulating T lymphocytes to develop immunity, and enhancing HIV-1 and SIV replication. For immunity, it is implied that DC-SIGN allows the DC to interact temporarily with naive T cells. Critical events of antigen recognition can then ensue, leading to the formation and function of a contact zone termed the immunological synapse, rich in interacting adhesion and signaling molecules. For immunodeficiency viruses, DC-SIGN enables the DC to bind and transmit virus to permissive T cells. This should occur in vivo beneath the genital epithelium and in the T cell areas of lymphoid tissues, because of DC-SIGN expression in these sites. DC-SIGN may allow DCs to carry additional pathogens to their cellular targets. The new data identify DC-SIGN as a potential site on DCs for manipulating both the immune response and HIV-1 infection. The results illustrate a larger issue. To understand and manipulate immune responsiveness, and many clinical areas involving the immune system, one should not restrict the analysis to antigens and lymphocytes. One must also consider DCs, the captivating controllers of immunity.

Acknowledgments

The author regrets that, because of space limitations, many valuable references could not be included.

Selected Reading

Adema, G.J., Hartgers, F., Verstraten, R., de Vries, E., Marland, G., Menon, S., Foster, J., Xu, Y., Nooyen, P., McClanahan, T., Bacon, K.B., and Figdor, C.G. (1997). Nature *387*, 713–717.

Bhardwaj, N., Young, J.W., Nisanian, A.J., Baggers, J., and Steinman, R.M. (1993). J. Exp. Med. *178*, 633–642.

Caux, C., Vanbervliet, B., Massacrier, C., Dezutter-Dambuyant, C., de Saint-Vis, B., Jacquet, C., Yoneda, K., Imamura, S., Schmitt, D., and Banchereau, J. (1996). J. Exp. Med. *184*, 695–706.

Cella, M., Sallusto, F., and Lanzavecchia, A. (1997). Curr. Opin. Immunol. *9*, 10–16.

Curtis, B.M., Scheerowske, S., and Watson, A.J. (1992). Proc. Natl. Acad. Sci. USA *89*, 8356–8360.

Davis, S.J., and van der Merwe, P.A. (1996). Immunol. Today *17*, 177–187.

Dhodapkar, M.V., and Bhardwaj, N. (2000). J. Clin. Immunol., in press.

Fayette, J., Dubois, B., Vandenabeele, S., Bridon, J.-M., Vanbervliet, B., Durand, I., Banchereau, J., Caux, C., and Briere, F. (1997). J. Exp. Med. *185*, 1909–1918.

Forster, R., Schubel, A., Breitfeld, D., Kremmer, E., Renner-Muller, I., Wolf, E., and Lipp, M. (1999). Cell *99*, 23–33.

Geijtenbeek, T.B.H., Torensma, R., van Vliet, S.J., van Duijnhoven, G.C.F., Adema, G.J., van Kooyk, Y., and Figdor, C.G. (2000a). Cell *100*, this issue, 575–585.

Geijtenbeek, T.B.H., Kwon, D.S., Torensma, R., van Vliet, S.J., van Duijnhoven, G.C.F., Middel, J., Cornelissen, I.L.M.H.A., Nottet, H.S.L.M., KewalRamani, V.N., Littman, D.R., Figdor, C.G., and van Kooyk, Y. (2000b). Cell *100*, this issue, 587–597.

Granelli-Piperno, A., Finkel, V., Delgado, E., and Steinman, R.M. (1999). Current Biol. *9*, 21–29.

Inaba, K., Turley, S., Iyoda, T., Yamaide, F., Shimoyama, S., Reis e Sousa, C., Germain, R.N., Mellman, I., and Steinman, R.M. (2000). J. Exp. Med., in press.

Inguili, E., Mondino, A., Khoruts, A., and Jenkins, M.K. (1997). J. Exp. Med. *185*, 2133–2141.

Josien, R., Hi, H.-L., Inguili, E., Sanna, S., Wong, B.R., Vologodskaia, M., Steinman, R.M., and Choi, Y. (2000). J. Exp. Med. *191*, 495–501.

Pope, M., Betjes, M.G.H., Romani, N., Hirmand, H., Cameron, P.U., Hoffman, L., Gezelter, S., Schuler, G., and Steinman, R.M. (1994). Cell *78*, 389–398.

Randolph, G.J., Beaulieu, S., Pope, M., Sugawara, I., Hoffman, L., Steinman, R., and Muller, W.A. (1998). Proc. Natl. Acad. Sci. USA *95*, 6924–6929.

Reece, J.C., Handley, A., Anstee, J., Morrison, W., Crowe, S.M., and Cameron, P.U. (1998). J. Exp. Med. *187*, 1623–1631.

Shaw, A.S., and Dustin, M.L. (1997). Immunity *6*, 361–369.

Stahl-Hennig, C., Steinman, R.M., Tenner-Racz, K., Pope, M., Stolte, N., Matz-Rensing, K., Grobschupff, G., Raschdorff, B., Hunsmann, G., and Racz, P. (1999). Science *285*, 1261–1265.

EVIDENCE APPENDIX F

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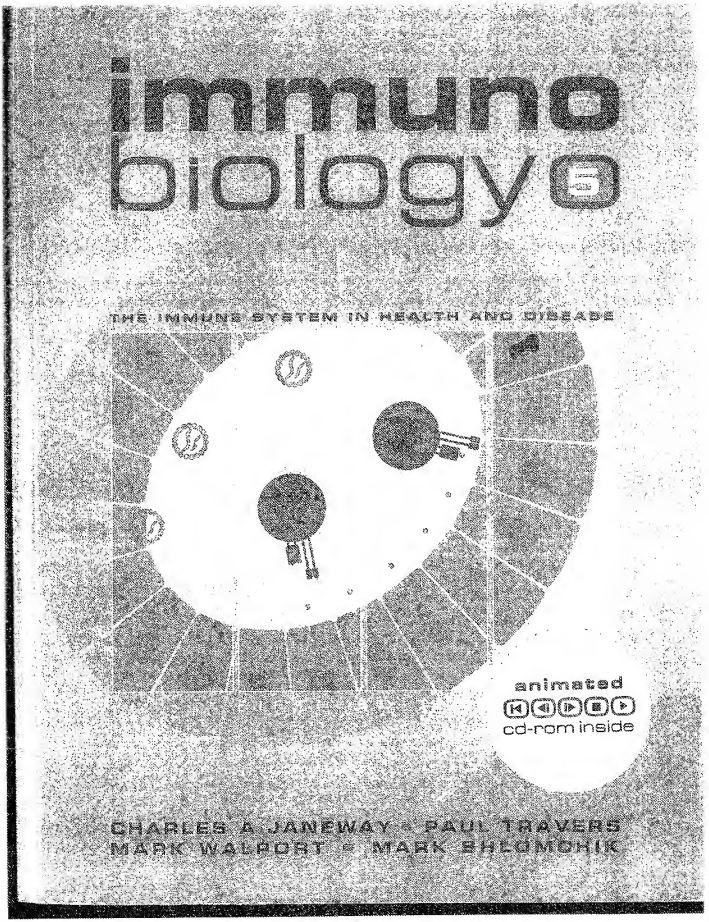
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immunobiology 5

THE IMMUNE SYSTEM IN HEALTH AND DISEASE

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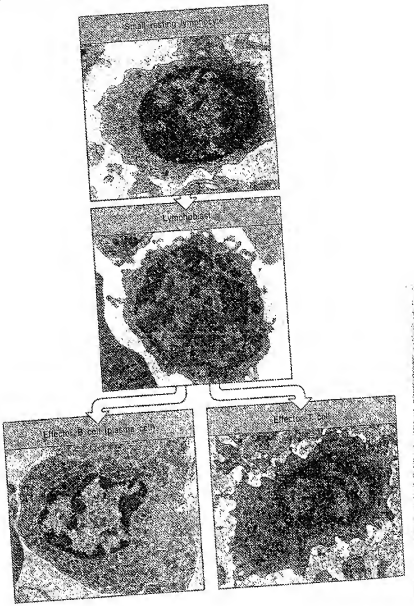

Fig. 1.19 Transmission electron micrographs of lymphocytes at various stages of activation to effector function. Small resting lymphocytes (top panel) have not yet encountered antigen. Note the scanty cytoplasm, the absence of rough endoplasmic reticulum, and the condensed chromatin, all indicative of an inactive cell. This could be either a T cell or a B cell. Small circulating lymphocytes are trapped in lymph nodes when their receptors encounter antigen on antigen-presenting cells. Stimulation by antigen induces the lymphocyte to become an active lymphoblast (center panel). Note the large size, the nucleoli, the enlarged nucleus with diffuse chromatin, and the active cytoplasm; again, T and B lymphoblasts are similar in appearance. This cell undergoes repeated division, which is followed by differentiation to effector function. The bottom panels show effector T and B lymphocytes. Note the large amount of cytoplasm, the nucleus with prominent nucleoli, abundant mitochondria, and the presence of rough endoplasmic reticulum, all hallmarks of active cells. The rough endoplasmic reticulum is especially prominent in plasma cells (effector B cells), which are synthesizing and secreting very large amounts of protein in the form of antibody. Photographs courtesy of N. Rooney.

1-13 Interaction with other cells as well as with antigen is necessary for lymphocyte activation.

Peripheral lymphoid tissues are specialized not only to trap phagocytic cells that have ingested antigen (see Sections 1-3 and 1-6) but also to promote their interactions with lymphocytes that are needed to initiate an adaptive immune response. The spleen and lymph nodes in particular are highly organized for the latter function.

All lymphocyte responses to antigen require not only the signal that results from antigen binding to their receptors, but also a second signal, which is delivered by another cell. Naive T cells are generally activated by activated dendritic cells (Fig. 1.21, left panel) but for B cells (Fig. 1.21, right panel), the

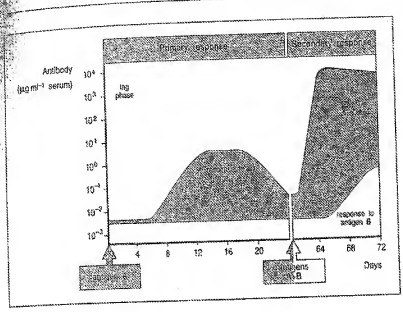

Fig. 1.20 The course of a typical antibody response. First encounter with an antigen produces a primary response. Antigen A introduced at time zero encounters little specific antibody in the serum. After a lag phase, antibody against antigen A (blue) appears: its concentration rises to a plateau, and then declines. When the serum is tested for antibody against another antigen, B (yellow), there is none present, demonstrating the specificity of the antibody response. When the animal is later challenged with a mixture of antigens A and B, a very rapid and intense secondary response to A occurs. This illustrates immunological memory, the ability of the immune system to make a second response to the same antigen more efficiently and effectively, providing the host with a specific defense against infection. This is the main reason for giving booster injections after an initial vaccination. Note that the response to B resembles the initial or primary response to A, as this is the first encounter of the animal with antigen B.

second signal is delivered by an armed effector T cell. Because of their ability to deliver activating signals, these three cell types are known as **professional antigen-presenting cells**, or often just **antigen-presenting cells**. They are illustrated in Fig. 1.22. Dendritic cells are the most important antigen-presenting cell of the three, with a central role in the initiation of adaptive immune responses (see Section 1-6). Macrophages can also mediate innate immune responses directly and make a crucial contribution to the effector phase of the adaptive immune response. B cells contribute to adaptive immunity by presenting peptides from antigens they have ingested and by secreting antibody.

Thus, the final postulate of adaptive immunity is that it occurs on a cell that also presents the antigen. This appears to be an absolute rule *in vivo*, although exceptions have been observed in *in vitro* systems. Nevertheless, what we are attempting to define is what does happen, not what can happen.

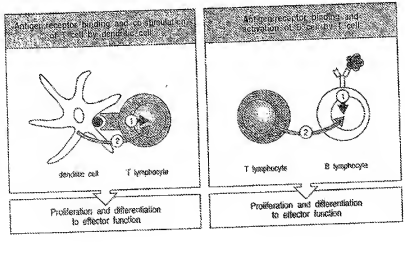

Fig. 1.21 Two signals are required for lymphocyte activation. As well as receiving a signal through their antigen receptor, mature naive lymphocytes must also receive a second signal to become activated. For T cells (left panel) it is delivered by a professional antigen-presenting cell such as the dendritic cell shown here. For B cells (right panel), the second signal is usually delivered by an activated T cell.

EVIDENCE APPENDIX G

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In Vivo Targeting of DC-SIGN-positive Antigen-presenting Cells in a Nonhuman Primate Model

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*Anke Kretz-Rommel, PhD,‡ Susan J. Faas, PhD,§ Carl G. Figdor, PhD,**
*and Gosse J. Adema, PhD**

Summary: In vivo targeting of antigen-presenting cells (APCs) with antigens coupled to antibodies directed against APC-specific endocytic receptors is a simple and a promising approach to induce or modulate immune responses against those antigens. In a recent in vitro study, we have shown that targeting of APCs with an antigen coupled to an antibody directed against the endocytic receptor DC-SIGN effectively induces a specific immune response against that antigen. The aim of the present study was to determine the ability of the murine antihuman DC-SIGN antibody AZN-D1 to target APCs in a cynomolgus macaque model after its administration in vivo. Immunohistochemical analysis demonstrated that macaques injected intravenously with AZN-D1 have AZN-D1–targeted APCs in all lymph nodes (LNs) tested and in the liver. DC-SIGN–positive cells were mainly located in the medullary sinuses of the LNs and in the hepatic sinusoids in the liver. No unlabeled DC-SIGN molecules were found in the LN of AZN-D1–injected macaques. Morphologic criteria and staining of sequential LN sections with a panel of antibodies indicated that the DC-SIGN–targeted cells belong to the myeloid lineage of APCs. In conclusion, this is the first study that shows specific targeting of APCs in vivo by using antibodies directed against DC-SIGN.

Key Words: anti-DC-SIGN antibody, cynomolgus macaque, antigen-presenting cells, lymph nodes, liver

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Dendritic cell (DC)-specific intracellular adhesion molecule-3-grabbing nonintegrin (DC-SIGN) is a mannose-specific type II membrane C-type lectin with a short amino-terminal cytoplasmic tail and a single carboxyl-terminal carbohydrate-recognition domain (CRD). DC-SIGN is expressed by macrophages and DCs.[1,2] Both cells types are antigen-presenting cells (APCs), which can capture and process antigens in the peripheral blood and tissues, migrate to the lymphoid tissue and present the antigens to resting T cells. DC-SIGN has been described as a receptor for several pathogens including HIV-1.[3] Importantly, DC-SIGN, and also another C-type lectin receptor DEC-205, can bind and endocytose antigens.[4–6] These antigens enter the endocytic compartments, are loaded onto major histocompatibility complex (MHC) class II molecules and stimulate proliferation of antigen-specific $CD4^+$ T cells.

The potent capacity of APCs to generate immune responses has led to the development of vaccination strategies that involve ex vivo loading of autologous APCs with tumor or pathogen-derived antigens and their subsequent administration to patients.[7,8] Unfortunately, this ex vivo approach is very laborious and expensive. An attractive alternative is to load the APCs with the antigen(s) directly in vivo. In principle, APCs can be targeted with antigens coupled to recombinant viral vectors, microparticles, receptor ligands, or receptor-specific antibodies. Targeting APCs in vivo with specific antibodies is an attractive method because of their specificity, applicability in the clinical setting, and because many antibodies that target a cell surface receptor can induce receptor-mediated endocytosis. Several studies have shown that antibodies directed against the endocytic receptor DEC-205 can target antigens to DCs in an in vivo mouse model[6,9–12] and, when combined with a maturation stimulus, can allow the induction of an immune response against those antigens. In a recent in vitro study, we have shown that targeting of keyhole limpet hemocyanin to human APCs via an antibody directed against the endocytic receptor DC-SIGN effectively induced the proliferation of keyhole limpet hemocyanin-specific $CD4^+$ and $CD8^+$ T cells.[5] Mice have 5 distinct DC-SIGN homologs that exhibit expression profiles distinct from that of human DC-SIGN, hampering the interpretation of in vivo studies in mice.[13] Therefore, the aim of this study was to determine the

specificity of the murine antihuman DC-SIGN monoclonal antibody AZN-D1 to target DC-SIGN-positive APCs in a nonhuman primate model in vivo. AZN-D1 has been shown previously to recognize rhesus macaque and chimpanzee DC-SIGN.[14] Because of the limited availability of rhesus macaques, we used cynomolgus macaques (*Macaca fascicularis*) that are genetically, physiologically, and behaviorally similar to rhesus macaques as a model. Therefore, we first demonstrated that cynomolgus macaques also express DC-SIGN, which is highly homologous to rhesus macaque and human DC-SIGN. We also showed that AZN-D1 could detect the expression of DC-SIGN in cynomolgus macaques. Finally, we evaluated the specificity of AZN-D1 for APCs in cynomolgus macaques in vivo.

MATERIALS AND METHODS

Antibodies

Preparations of AZN-D1 and AZN-D3 (mIgG1, antihuman DC-SIGN)[1] were produced from hybridoma supernatants, or from ascites fluid after a 1:5 dilution with Pierce Binding Buffer (Pierce, Rockford, IL). Briefly, diluted antibody was loaded onto Protein A Sepharose 4 Fast Flow columns (Amersham, Buckinghamshire, UK), the columns washed with binding buffer, the bound antibody eluted with 100 mM of glycine, pH 3.5 and neutralized with Tris buffer. Absorbance was determined at 280 nm and chosen fractions were pooled before dialysis into phosphate-buffered saline (PBS). The overall average yield was 3.9 mg of purified antibody/mL initial ascites fluid. AZN-D1 was biotinylated with EZ-Link Sulfo-NHS-LC-Biotin (Pierce) according to the manufacture's protocol. The rabbit anti-CD3 polyclonal sera, mouse monoclonal anti-CD20 (clone L26, mIgG1), and anti-CD68 (clone EBM11, mIgG1) were purchased from DAKO Cytomation, Glostrup, Denmark). Mouse monoclonal anti-DC-LAMP (clone 104.G4) and biotinylated mouse monoclonal anti-HLA-DR/DP (MHC class II; clone Q5/13) were purchased by Immunotech (Marseille, France) and Leinco Technologies (St Louis, MO), respectively. Mouse monoclonal anti-CD31 (clone WM-59) was purchased from Pharmingen (San Jose, CA). Mouse IgG1 and IgG2a (R&D systems, Abingdon, UK) and total rabbit IgG (Jackson ImmunoResearch; Westgroove, PA) were used as controls. Biotinylated horse antimouse and biotinylated goat antirabbit were purchased by Vector Laboratories (Burlingame, CA).

Animal and Human Tissues

Nine cynomolgus macaques (*M. fascicularis*) were used in this study. The experiments described were performed using a minimal number of nonhuman primates, in accordance with ethical policies established by the United Kingdom Animals (Scientific Procedures) Act (1986) (the Act) and the associated Codes of Practice for the Housing and Care of Animals used in Scientific Procedures and the Humane Killing of Animals under Schedule 1 to the Act, issued under Section 21 of the Act. Eight cynomolgus macaques were purpose-bred and obtained from Vietnam via a commercial supplier (Belgrave Services). The experiments were performed at Huntingdon Life Sciences, Woolley Road, Alconbury, Huntingdon, Cambridgeshire, England. The in-life experimental procedures were subject to the provisions of the United Kingdom Animals (Scientific Procedures) Act (1986) (the Act); and the associated Codes of Practice for the Housing and Care of Animals used in Scientific Procedures and the Humane Killing of Animals under Schedule 1 to the Act, issued under Section 21 of the Act. Eight animals were treated intracutaneously with 2.5% oxazolone on days 3, 4, and 5. Oxazolone is a chemical allergen, which can maximize the migration of local APCs from the injection site to the draining (auricular) lymph node (LN). Four of these macaques also received intravenous injections of 3 mg/kg/d of AZN-D1 on days 1 to 7. The animals were euthanized on day 8 with a lethal dose of pentobarbitone anesthesia and exsanguinated by excision of the carotid blood vessels. Tissues were collected immediately after euthanasia and specified organs were fresh-frozen. Frozen samples were placed on corks, snap frozen in isopentane, cooled in liquid nitrogen, and stored frozen (approximately −70°C). For comparison, frozen inguinal LN and liver tissues from an untreated (naive) macaque were obtained from the German Primate Center (Göttingen, Germany) euthanized owing to poor prognosis after trauma. This untreated control animal was of similar health and age and was housed under similar conditions as the 8 animals described above. Animal care and handling was performed under the German Animal Protection Law and in accordance with guidelines of the German Primate Center (Göttingen, Germany). As the expression pattern of APCs in the LNs of untreated macaques was similar to those observed in the LNs of macaques that treated with oxazolone, both untreated and oxazolone-treated macaques will be referred to as control cynomolgus macaques.

A sample of human liver was obtained from 1 patient, a 78-year-old man undergoing a partial hepatic resection as treatment for colon carcinoma, following national guidelines regarding the use of human tissues. All tissues were sectioned at 5 μm for immunohistochemistry and eosin and hematoxylin staining.

Cloning and Sequencing of Cynomolgus Macaque DC-SIGN

Frozen liver and LNs tissues from control cynomolgus macaques were homogenized and lysed in 1 mL TRIzol (Invitrogen Life Technologies, Gaithersburg, MD) according to the manufacturer's guidelines. Total RNA was dissolved in diethylpyrocarbonate-treated water and 12 μg of RNA were used for the synthesis of complementary DNA (cDNA). Afterwards, a polymerase chain reaction (PCR) was performed with the following primers: 5'-GTGCTGAGGAGCAGAACTTC-3' (sense) and 5'-GCAGATCCAGAATTTGGCAAG-3' (antisense). These primers were complementary to regions

conserved between the human and rhesus DC-SIGN genes. cDNA was subjected to 35 cycles to detect DC-SIGN mRNA. Amplified DNA was extracted and purified from a standard agarose gel in TAE (0.04 M of Tris, 0.001 M of EDTA-Na2-salt, and 0.02 M of acetic acid) buffer with a QIAquick Gel Extraction Kit (QIAGEN, Valencia, CA) according to the manufacturer's guidelines. The PCR product was then inserted into a pGEM-T Easy Vector (Promega, Madison, WI) according to the manufacturer's guidelines and DH5α competent bacteria were transformed with the construct by heat-shock. Positive clones were selected by PCR using the same primers as described above. Plasmid DNA was purified from clones that contained the insert using a QIAprep Miniprep (QIAGEN) according to the manufacturer's guidelines. Sequence analysis was performed on 400 ng of plasmid DNA using 50 ng of T7 promotor or M13 reverse primers.

Immunohistochemistry

Cryosections of tissues were fixed at room temperature (RT) with either cold 100% acetone for 10 minutes or 4% paraformaldehyde for 30 minutes, depending on the primary antibody used. After washing with PBS, tissue sections were incubated for 1 hour at RT with 10% normal serum from the species in which the second antibody was raised. The serum was then discarded and incubated overnight at 4°C with the primary antibody. For the liver tissue samples, endogenous biotin was blocked by 15-minute incubation with avidin, followed by 15 minutes incubation with biotin (Avidin/Biotin Blocking kit, Vector Laboratories, Burlingame, CA). Liver tissue sections were washed with PBS before and after the biotin block. Afterwards the tissue sections were washed with PBS and incubated with the secondary antibody for 30 minutes at RT. After extensive washing with PBS, the final staining was performed with the ABC-PO/ABC-AP Vectastain kit (Vector Laboratories, Burlingame, CA). AEC (Zymed, Invitrogen Immunodetection, San Francisco, CA) and diaminobenzidine (ImmunoLogic, Duiven, The Netherlands) were used as substrates for peroxidase and Fast Red (Sigma-Aldrich, Zwijndrecht, The Netherlands) was used as substrate for alkaline phosphatase. Tissue sections were counterstained with hematoxylin and washed extensively with water and either mounted with Kayser's glycerol (Merck, Darmstadt, Germany) or, when diaminobenzidine substrate was used, were dehydrated and mounted with Entellan (J.T. Baker, Chemicals BV, Deventer, The Netherlands).

RESULTS

Cynomolgus Macaque DC-SIGN CRD Is Highly Homologous to Rhesus Macaque and Human DC-SIGN

As cynomolgus macaque DC-SIGN had not been previously sequenced, and as the epitope recognized by AZN-D1 contains a valine residue at position 351 (V351) of the human DC-SIGN CRD, we amplified the cynomolgus macaque DC-SIGN CRD from LN and liver tissues with primers complementary to regions conserved between the human and rhesus DC-SIGN genes. Sequence analysis of the protein sequence from cynomolgus macaque DC-SIGN CRD indicated that it is 100%, 98%, 97%, and 79% homologous to pig-tailed macaque, rhesus macaque, human DC-SIGN, and human L-SIGN, respectively (Table 1). In addition, the CRD region containing V351 is conserved in cynomolgus macaque but not in rhesus macaque DC-SIGN, suggesting that AZN-D1 can bind to cynomolgus macaque DC-SIGN.

High Expression of DC-SIGN in Lymphoid Tissues of Cynomolgus Macaques

Although the expression of DC-SIGN in rhesus macaques and chimpanzees has been extensively described,[14–16] the expression of DC-SIGN in cynomolgus macaques has not. Therefore, we first analyzed the expression of DC-SIGN on frozen LN sections from control cynomolgus macaques. In addition, antibodies against CD20 (Fig. 1A) and CD3 (Fig. 1B) were used to stain B cells and T cells, respectively. Isotype-matched controls did not demonstrate significant background staining (data not shown). Similar to findings in rhesus macaques,[15] large numbers of DC-SIGN–positive cells were present in the medullary sinuses and some DC-SIGN–positive cells were present in the afferent sinuses and in the paracortical area of several LNs from control cynomolgus macaques (Figs. 1D–H).

TABLE 1. Comparison of the Protein Sequence of Human, Rhesus Macaque, Pig-tailed Macaque, and Cynomolgus Macaque DC-SIGN CRD

Cynomolgus macaque	AEEQNFLQLQSSRSNRFTWMGLSDLNHEGTWQWVDGSPLLPSFKQYWNKGEPNNVGEEDC	60
Pig-tailed macaque	AEEQNFLQLQSSRSNRFTWMGLSDLNHEGTWQWVDGSPLLPSFKQYWNKGEPNNVGEEDC	60
Rhesus macaque	AEEQNFLQLQSSRSNRFTWMGLSDLNHEGTWQWVDGSPLLPSFKQYWNKGEPNNVGEEDC	60
Human	AEEQNFLQLQSSRSNRFTWMGLSDLNQEGTWQWVDGSPLLPSFKQYWNRGEPNNVGEEDC	60
	* *	
Cynomolgus macaque	AEFSGNGWNDDKCNLAKFWIC	81
Pig-tailed macaque	AEFSGNGWNDDKCNLAKFWIC	81
Rhesus macaque	AEFSGNGWNDDKCNLAKFWIC	81
Human	AEFSGNGWNDDKCNLAKFWIC	81
	* *	

The solid bar indicates residue Val351, which is crucial for AZN-D1 binding to DC-SIGN.

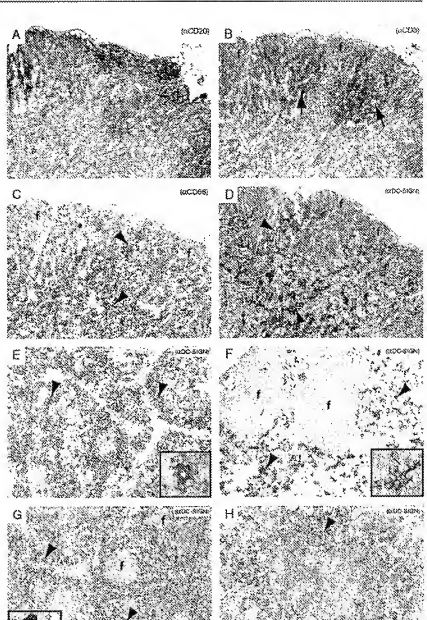

FIGURE 1. Expression patterns of (A) CD20 in B-cell follicles, (B) CD3 in paracortical areas, (C) CD68 on sinus cells, and (D–H) DC-SIGN in frozen serial sections of inguinal (A–D), auricular (E, F), or mesenteric (G–H) LNs from control cynomolgus macaques. Tissue sections were stained with the antibodies described above followed by biotinylated horse antimouse antibody. Magnification: 50 × (A–D); 100 × (G); 200 × (E, F, H); 630 × (insets). Arrowheads indicate sinuses, "f", follicles; arrows, paracortical areas.

The DC-SIGN–positive cells are large and irregular in shape, consistent with the morphology of myeloid cells (insets in Figs. 1E–G). Subsequent staining of consecutive sections with the myeloid marker CD68 (Fig. 1C) indicated that most DC-SIGN–positive cells in the sinuses and on the paracortical region are of myeloid origin. As expected, CD68 staining revealed a broader expression pattern than only DC-SIGN–positive cells. Next, sequential sections of the same auricular LNs were stained with antibodies directed against DC-SIGN (Figs. 2A, C), the mature DC marker DC-LAMP (Fig. 2B) or against MHC class II (Fig. 2D), which is present on both mature and immature DCs, and also B cells, interdigitating DCs and macrophages. The anti-DC-LAMP antibody stained only a few cells in the paracortical region and medullary sinuses (Fig. 2B) and the anti-MHC-II antibody labeled a large number of cells distributed throughout the LN (Fig. 2D). In summary, as previously described for rhesus macaques,[15] the expression pattern of DC-SIGN correlated well with that of CD68, but was less consistent with the expression patterns of the mature DC marker DC-LAMP or of MHC class II. These findings suggest that cynomolgus macaque DC-SIGN is

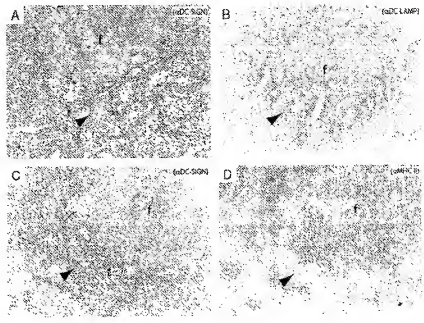

FIGURE 2. Expression patterns of (A, C) DC-SIGN on sinus cells, (B) DC-LAMP on scattered cells outside the follicles, (D) MHC class II in follicles in frozen serial sections of auricular LNs from control cynomolgus macaques. Tissue sections were stained with the antibodies described above followed by biotinylated horse antimouse antibody. Magnification: 100 ×. Arrowheads indicate sinuses; "f", follicles.

expressed on myeloid lineage cells such as macrophages and/or immature DCs.

Intravenous Administration of Anti-DC-SIGN Antibody Targets DC-SIGN–positive Cells in Lymphoid Tissues of Cynomolgus Macaques In Vivo

To determine whether and to what extent AZN-D1 could specifically target DC-SIGN–positive cells in vivo, cynomolgus macaques were treated with AZN-D1 delivered by intravenous injection. As it has previously been shown that mIgG1 delivered by intravenous injection (2 mg/kg/d for 12 consecutive days) into cynomolgus monkeys did not result in the detection of mIgG1-bound mononuclear cells within the LNs,[17] we did not euthanize more animals to include this control arm in our study. As subcutaneous injections only reach the draining LNs, AZN-D1 was delivered by intravenous injections to reach as many LNs as possible. Sections from several LNs of these macaques were stained with biotinylated horse antimouse antibody to assess the targeting efficiency of AZN-D1 in these tissues. Sections of auricular LNs showed massive numbers of AZN-D1–targeted cells (Figs. 3A–D). These AZN-D1–targeted cells were mainly present in the medullary sinuses, but were also evident in the afferent sinuses and in the paracortical area (Figs. 3A–D and Table 2). Sections of mesenteric LNs from cynomolgus macaques that were treated with AZN-D1 also showed numerous AZN-D1–targeted cells (Figs. 3E, F). Biotinylated horse antimouse antibody staining of sections from an untreated macaque did not demonstrate any background staining (data not shown).

To assess the percentage of DC-SIGN molecules that were targeted by AZN-D1, LN sections from cynomolgus macaques treated with AZN-D1 were stained with biotinylated AZN-D1. This biotinylated AZN-D1 antibody is as effective as unlabeled AZN-D1 in detecting DC-SIGN–positive cells in macaques (Figs. 4A, B), and allows visualization of the DC-SIGN molecules that remain unlabeled after the intravenous AZN-D1 administration. Strikingly, upon staining of sections of LNs from AZN-D1–treated macaques with biotinylated AZN-D1, no reactivity with the biotinylated antibody was observed (Fig. 4C). This indicates that essentially all DC-SIGN molecules present in these LNs have been targeted by the intravenously injected AZN-D1 antibody. Staining of LNs from AZN-D1–treated macaques with unlabeled AZN-D1 followed by biotinylated horse antimouse antibody (Fig. 4D) resulted in a similar staining pattern as observed with biotinylated horse antimouse antibody alone (Figs. 3A–D), confirming that all DC-SIGN molecules present in the LNs are targeted by AZN-D1.

Anti-DC-SIGN Antibody Targets Kupffer Cells in the Liver of Cynomolgus Macaques

Liver/LN-specific intracellular adhesion molecule-3-grabbing nonintegrin (L-SIGN or DC-SIGNR) is 77% identical to DC-SIGN, has a similar 3-dimensional structure and shares similar ligands. However, L-SIGN is expressed in specialized sinusoidal endothelial cells of the liver, LNs, and placenta, but not in DCs or macrophages. Sections of liver from cynomolgus macaques that were treated with AZN-D1 were stained with biotinylated horse antimouse antibody to assess the specificity of AZN-D1 in these tissues. Surprisingly, numerous AZN-D1–targeted cells were observed in the

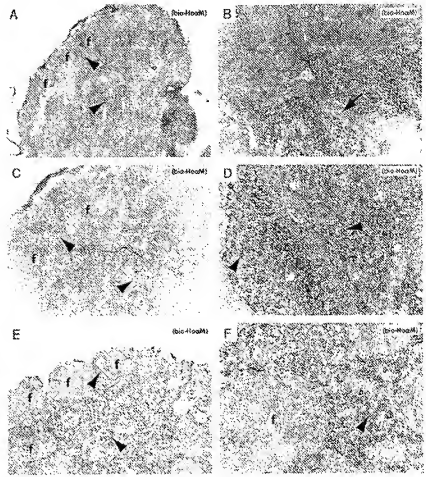

FIGURE 3. Cynomolgus macaques administered AZN-D1 in vivo have AZN-D1–targeted cells within (A–D) auricular and (E, F) mesenteric LNs. Frozen tissue sections were only stained with biotinylated horse antimouse antibody (bio-HoaM). Magnification: 50 × (A, E); 100 × (B, C); 200 × (D, F). Arrowheads indicate sinuses; "f", follicles; arrow, the hilum.

hepatic sinusoids in the liver of these animals (Fig. 5). To determine which cell types were targeted by AZN-D1, serial sections of livers from control cynomolgus macaques were stained with antibodies against human DC-SIGN (AZN-D1, Fig. 6A), DC-SIGN/L-SIGN (AZN-D3, Fig. 6B), CD68 (expressed on Kupffer cells or liver macrophages) (Fig. 6C), and CD31 (expressed on liver sinusoidal endothelial cells (LSECs) (Fig. 6D). The staining patterns of DC-SIGN and L-SIGN (obtained with AZN-D1 and AZN-D3) were similar to that observed after labeling with biotinylated horse antimouse antibody alone (Fig. 5). Interestingly, these staining

TABLE 2. Distribution of In Vivo-targeted AZN-D1 in the Draining LNs of Cynomolgus Macaques

Cynomolgus macaque	Treatment	Lymphoid Follicles	Paracortical Area	Medullary Sinuses	Afferent Sinuses
1	None	–	–	–	–
2	Oxazolone	–	–	–	–
3	Oxazolone	–	–	–	–
4	Oxazolone	–	–	–	–
5	Oxazolone	–	–	–	–
6	Oxazolone + AZN-D1	–	+	+ + +	+ +
7	Oxazolone + AZN-D1	–	+	+ + +	+ +
8	Oxazolone + AZN-D1	–	+	+ + +	+ +
9	Oxazolone + AZN-D1	–	+	+ + +	+ +

Animal 1 received no treatment. Animals 2 to 9 were treated intracutaneously with 2.5% oxazolone on days 3 to 5. Animals 6 to 9 also received intravenous injections of 3 mg/kg/d of AZN-D1 on days 1 to 7. The animals were euthanized on day 8. Frozen tissue sections from the draining (auricular) LNs of these macaques were stained with biotinylated horse antimouse antibody. Average staining intensity and extent of staining were scored: "–", negative; "+", < 5% positive cells; "+ +", 5% to 40% positive cells; "+ + +", > 40% positive cells.

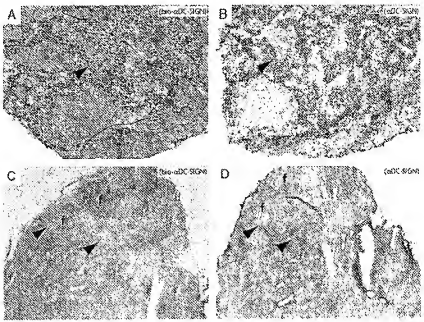

FIGURE 4. Unlabeled DC-SIGN molecules in the auricular LNs of cynomolgus macaques administered AZN-D1 in vivo. Serial frozen tissue sections from control cynomolgus macaques (A, B) or cynomolgus macaques treated with AZN-D1 (C, D) were stained with biotinylated AZN-D1 (bio-αDC-SIGN) alone (A, C) or unlabeled AZN-D1 (αDC-SIGN) followed by biotinylated horse antimouse antibody (B, D). Magnification: 50 × (C, D); 100 × (A, B). Arrowheads indicate sinuses; "f", follicles.

patterns were most consistent with that of anti-CD68 but not with the staining pattern of anti-CD31. These results are in contrast to those observed on human liver, in which AZN-D1 labels very few liver DCs (Fig. 7A) and AZN-D3 labels LSECs (Fig. 7B). As expected, staining of human liver with antibodies against CD68 (Fig. 7C) and CD31 (Fig. 7D) labeled Kupffer cells and LSECs, respectively. These results suggest that AZN-D1 is targeting Kupffer cells in the liver of cynomolgus macaques.

DISCUSSION

To our knowledge, this is the first study that demonstrates in vivo targeting of DC-SIGN–positive cells after the administration of an anti-DC-SIGN antibody. As expected, we have shown that cells targeted with the antihuman DC-SIGN monoclonal antibody AZN-D1 demonstrate the same distribution pattern in LNs as DC-SIGN–positive cells, which indicates that intravenous injection of AZN-D1 specifically targeted DC-SIGN–positive cells. These cells are mainly located in the LN medullary sinuses, although some cells can also be found in the afferent sinuses and on the paracortical or T-cell areas. As immunophenotyping of the DC-SIGN–positive cells in cynomolgus macaques is hampered by the absence of DC-specific or macrophage-specific antibodies, it remains difficult to unequivocally distinguish cynomolgus macaque DCs from macrophages. Although some studies have shown that DC-SIGN is mainly expressed by DCs,[1,3,18] recent studies have shown that DC-SIGN is expressed on medullary sinus macrophages in the paracortical area of normal human and rhesus macaque LNs.[2,15] Since several chimpanzee DC-SIGN alleles have been described,[19] it is tempting to speculate that human or cynomolgus macaque DC-SIGN alleles may also exist and that these alleles may account for the differences observed between different studies. Future studies are needed to clarify this hypothesis. Notwithstanding, both DCs and macrophages are APCs that can induce powerful and specific immune responses against foreign antigens and also induce cross-presentation of exogenous antigens on MHC class I molecules.[20–22] Irrespective of the precise

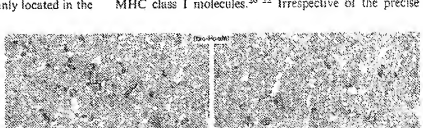

FIGURE 5. Cynomolgus macaques administered AZN-D1 in vivo have AZN-D1–targeted cells in the liver. Frozen tissue sections were stained with biotinylated horse antimouse antibody (bio-HorseAb). Two representative sections are shown. Magnification: 400 ×.

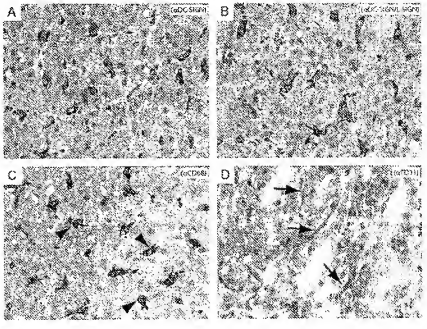

FIGURE 6. Frozen serial sections of liver tissue from control cynomolgus macaques were stained with (A) AZN-D1 (αDC-SIGN), (B) AZN-D3 (αDC-SIGN/L-SIGN), (C) anti-CD68 antibody for Kupffer cells, or with (D) anti-CD31 antibody for LSECs followed by biotinylated horse antimouse antibody. Magnification: 400 ×. Arrowheads indicate Kupffer cells; arrows, LSECs.

identity of these DC-SIGN–positive cells, our data demonstrate that DC-SIGN–positive APCs can be efficiently targeted in vivo with an antihuman DC-SIGN–specific mouse monoclonal antibody. Essentially all DC-SIGN molecules present in the LNs of AZN-D1–treated macaques were targeted with this antibody using the described intravenous treatment regimen. Others have shown that intravenous administration of mIgG1 (2 mg/kg/d for 12 consecutive days) into cynomolgus monkeys does not result in the detection of the mouse antibody on LN mononuclear cells.[17] Therefore, it seems unlikely that AZN-D1 treatment resulted in nonspecific uptake of the antibody or a down-regulation of DC-SIGN molecules on APCs in the LNs. Although DC-SIGN is a recycling receptor and the macaques were euthanized 24 hours after the last dose of AZN-D1, the administered dose of AZN-D1 (3 mg/kg/d) for 7 days resulted in a plasma concentration of AZN-D1 that was high enough to occupy all DC-SIGN molecules for 24 hours after the last dose. This dose of AZN-D1 was used

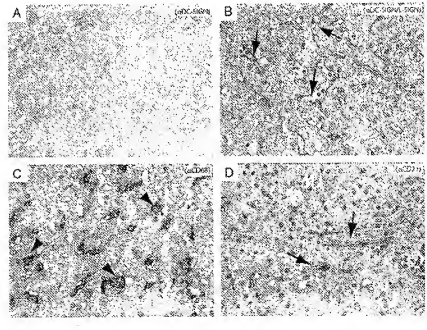

FIGURE 7. Frozen serial sections of liver tissue from a human biopsy were stained with (A) AZN-D1 (αDC-SIGN), (B) AZN-D3 (αDC-SIGN/L-SIGN), (C) anti-CD68 antibody for Kupffer cells, or with (D) anti-CD31 antibody for LSECs followed by biotinylated horse antimouse antibody. Magnification: 400 ×. Arrowheads indicate Kupffer cells; arrows, LSECs.

as a proof-of-principle to show that anti-DC-SIGN antibodies can target a significant number of DC-SIGN molecules in vivo and can reach the LNs. Subsequent in vivo studies can now be designed to determine the immunologic consequences of targeting DC-SIGN in cynomolgus macaques. We note that no apparent pathologic effects were observed in this study after a short-term administration of AZN-D1, but this should be further evaluated in additional studies in which antibody dose and frequency of administration are systematically examined.

As the DC-SIGN homolog L-SIGN is mainly expressed on LSECs in humans, we also assessed the specificity of AZN-D1 for APCs in the liver of cynomolgus macaques. Surprisingly, in contrast to results using human liver in which only a few scattered APCs stained with AZN-D1, immunohistochemical analysis of the reactivity of AZN-D1 on liver sections from control cynomolgus macaques revealed numerous labeled cells. As previously described for Rhesus macaques,[15] the staining pattern observed with AZN-D1 was similar to that of an anti-CD68 antibody, which labels the liver macrophages (Kupffer cells). Similarly, the anti-L-SIGN antibody AZN-D3 did not label LSECs, as was observed in the case of human liver, but rather, labeled cells with a very different morphology. Analysis of the AZN-D3 staining pattern was also very different from the staining pattern observed with an anti-CD31 antibody, which labeled LSECs in both human and control cynomolgus macaque liver. This fundamental difference in the liver staining pattern between human and both cynomolgus and rhesus monkeys may be either due to structural differences in the L-SIGN/DC-SIGN proteins themselves or to different expression patterns of DC-SIGN and L-SIGN in cynomolgus macaque liver compared with human liver. As AZN-D1 is capable of recognizing DC-SIGN in the LNs of these macaques, it is reasonable to assume that the antibody specificity is the same, but the expression patterns of DC-SIGN and L-SIGN in the liver of cynomolgus macaques are different from human liver.[16]

In agreement with these observations, cynomolgus macaques treated with AZN-D1 had AZN-D1–targeted cells in their livers. As was seen in control cynomolgus macaques, the staining pattern of these AZN-D1–targeted cells closely correlated with the distribution of Kupffer cells, as revealed by reactivity with an anti-CD68 antibody. It is unlikely that the AZN-D1 labeling in vivo is due simply to nonspecific binding of the antibody to Fc receptors on Kupffer cells, as liver sections from control macaques did not show Kupffer cell staining using an isotype-matched antibody control (data not shown), but did show reactivity with both anti-DC-SIGN and anti-L-SIGN antibodies. Instead, our results suggest that AZN-D1 is binding to a DC-SIGN homolog on Kupffer cells in cynomolgus monkeys.

In conclusion, this study provides proof-of-principle that an anti-DC-SIGN monoclonal antibody administered in vivo is capable of targeting APCs in LNs and in the liver. Recent in vitro studies have already shown that targeting antigens to APCs via a humanized anti-DC-SIGN antibody effectively induces antigen-specific immune responses,[5] and that MHC class II-restricted helper peptides and proteins targeted through DC-SIGN generate primary immune responses (Anke Kretz-Rommel, unpublished results). As has been described previously for targeting of DCs with anti-DEC-205 antibodies in mice,[9,10,12] future in vivo immunotherapeutic studies with anti-DC-SIGN antibodies, including humanized anti-DC-SIGN antibodies,[5] should explore the effect of APC maturation signals on induction of immunity or tolerance against the antigens targeted via DC-SIGN.

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REFERENCES

1. Geijtenbeek TB, Torensma R, van Vliet SJ, et al. Identification of DC-SIGN, a novel dendritic cell-specific ICAM-3 receptor that supports primary immune responses. *Cell.* 2000;100:575–585.
2. Grandal-Piperno A, Pritsker A, Pack M, et al. Dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin CD209 is abundant on macrophages in the normal human lymph node and is not required for dendritic cell stimulation of the mixed leukocyte reaction. *J Immunol.* 2005;175:4265–4273.
3. Geijtenbeek TB, Kwon DS, Torensma R, et al. DC-SIGN, a dendritic cell-specific HIV-1-binding protein that enhances trans-infection of T cells. *Cell.* 2000;100:587–597.
4. Mahnke K, Guo M, Lee S, et al. The dendritic cell receptor for endocytosis, DEC-205, can recycle and enhance antigen presentation via major histocompatibility complex class II-positive lysosomal compartments. *J Cell Biol.* 2000;151:673–684.
5. Tacken PJ, de Vries IJ, Gijzen K, et al. Effective induction of naive and recall T-cell responses by targeting antigen to human dendritic cells via a humanized anti-DC-SIGN antibody. *Blood.* 2005;106:1278–1285.
6. Guo M, Gong S, Maric S, et al. A monoclonal antibody to the DEC-205 endocytosis receptor on human dendritic cells. *Hum Immunol.* 2000;61:729–738.
7. de Vries IJ, Lesterhuis WJ, Scharenborg NM, et al. Maturation of dendritic cells is a prerequisite for inducing immune responses in advanced melanoma patients. *Clin Cancer Res.* 2003;9:5091–5100.
8. Lu W, Arraes LC, Ferreira WT, et al. Therapeutic dendritic-cell vaccine for chronic HIV-1 infection. *Nat Med.* 2004;10:1359–1365.
9. Bonifaz L, Bonnyay D, Mahnke K, et al. Efficient targeting of protein antigen to the dendritic cell receptor DEC-205 in the steady state leads to antigen presentation on major histocompatibility complex class I products and peripheral CD8+ T cell tolerance. *J Exp Med.* 2002;196:1627–1638.
10. Hawiger D, Inaba K, Dorsett Y, et al. Dendritic cells induce peripheral T cell unresponsiveness under steady state conditions in vivo. *J Exp Med.* 2001;194:769–779.
11. Mahnke K, Qian Y, Knop J, et al. Induction of CD4+ /CD25+ regulatory T cells by targeting of antigens to immature dendritic cells. *Blood.* 2003;101:4862–4869.
12. Bonifaz LC, Bonnyay DP, Charalambous A, et al. In vivo targeting of antigens to maturing dendritic cells via the DEC-205 receptor improves T cell vaccination. *J Exp Med.* 2004;199:815–824.
13. Park CG, Takahara K, Umemoto E, et al. Five mouse homologues of the human dendritic cell C-type lectin, DC-SIGN. *Int Immunol.* 2001;13:1283–1290.

14. Geijtenbeek TB, Koopman G, van Duijnhoven GC, et al. Rhesus macaque and chimpanzee DC-SIGN act as HIV/SIV gp120 transreceptors, similar to human DC-SIGN. *Immunol Lett*. 2001;79:161–167.
15. Sekaly RP, Alvarez X, Lackner AA. Distribution and immunophenotype of DC-SIGN-expressing cells in SIV-infected and uninfected macaques. *AIDS Res Hum Retroviruses*. 2002;18:1021–1029.
16. Bashirova AA, Wu L, Cheng J, et al. Novel member of the CD209 (DC-SIGN) gene family in primates. *J Virol*. 2003;77:217–227.
17. Cesmi AB, Conti D, Debrosicu FL, et al. In vivo effects of monoclonal antibody to ICAM-1 (CD54) in nonhuman primates with renal allografts. *J Immunol*. 1990;144:4604–4612.
18. Engering A, van Vliet SJ, Hebeda K, et al. Dynamic populations of dendritic cell-specific ICAM-3 grabbing nonintegrin-positive immature dendritic cells and liver/lymph node-specific ICAM-3 grabbing nonintegrin-positive endothelial cells in the outer zones of the paracortex of human lymph nodes. *Am J Pathol*. 2004;164:1587–1595.
19. Samos PR, Michel-Salzat A, Bator C. Chimpanzee DC-SIGN alleles predict the existence of A and B isoforms, but do not support a role for resistance to HIV infection. *AIDS Res Hum Retroviruses*. 2005;21:820–829.
20. Brode S, Macary PA. Cross-presentation: dendritic cells and macrophages bite off more than they can chew! *Immunology*. 2004;112:345–351.
21. Pozzi LA, Maciaszek JW, Rock KL. Both dendritic cells and macrophages can stimulate naive CD8T cells in vivo to proliferate, develop effector function, and differentiate into memory cells. *J Immunol*. 2005;175:2071–2081.
22. Kovacsovics-Bankowski M, Rock KL. Presentation of exogenous antigens by macrophages: analysis of major histocompatibility complex class I and II presentation and regulation by cytokines. *Eur J Immunol*. 1994;24:2421–2428

(xi). RELATED PROCEEDINGS APPENDIX

None.